UNIVERSITY OF ALBERTA

DIETARY FAT, PLASMA TRIGLYCERIDES AND DE NOVO LIPOGENESIS IN TYPE 2 DIABETES

by

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ABSTRACT

Diabetes predisposes individuals to a high risk of cardiovascular disease. Perturbations in carbohydrate and fat processing in the body lead to detrimental effects on triglyceride metabolism, but the underlying mechanisms are not fully understood. Reduction in fat intake is often recommended to lower cardiovascular disease risk. However, decreasing fat and increasing carbohydrate intake consistently raises plasma triglyceride levels, an independent risk factor for the disease.

The first study tested whether isocaloric replacement of carbohydrate with monounsaturated fat would reduce triglyceride levels and hepatic de novo lipogenesis in free-living type 2 diabetic and matched non-diabetic subjects. Fat intake was increased by adding olive oil to the usual diet, while decreasing carbohydrate-rich foods. This reduced plasma triglycerides in a subgroup of subjects with higher triglycerides but did not reduce fatty acid synthesis. However, a relationship was observed between fatty acid synthesis and plasma triglycerides in non-diabetic subjects that was not observed in type 2 diabetes subjects.

The second study examined whether diabetes and/or lower fat intake would result in higher fasting and postprandial triglycerides and if this was associated with de novo lipogenesis or plasma free fatty acids. A short-term lower fat diet was compared to a higher fat/high oleate diet in type 2 diabetic and matched non-diabetic control subjects in a blinded randomized crossover design. The amount of total and individual fatty acids in VLDL-triglyceride, free fatty acids, as well as hepatic and extra-hepatic de novo fatty acid was estimated. Deuterium incorporation techniques were used for the first time in humans

to measure de novo lipogenesis of specific fatty acids. Diabetes and insulin resistance tended to affect aspects of fasting and postprandial concentration, whereas diet seemed to influence fatty acid composition and synthesis. Higher fat intake generally decreased de novo lipogenesis and did not increase postprandial triglycerides. Synthesis of both total and individual fatty acids varied greatly between subjects and diets. Lipogenesis potentially affects the amount of circulating saturated fatty acids in the body. Whether a difference in lipogenic capacity and/or "quality" contributes to hypertriglyceridemia, atherogenesis and other metabolic alterations that occur in diabetes requires further investigation.

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I dedicate this thesis to the memory of my mother, who was my biggest fan
... and I hers.

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LIST OF ABBREVIATIONS

14:0	C14:0, myristic acid, myristate
16:0	C16:0, palmitic acid, palmitate
16:1	C16-1n-7, palmitoleic acid, palmitoleate
18:0	C18:0, stearic acid, stearate
18:1	C18:1n-9, oleic acid, oleate
18:2	C18:2n-6, linoleic acid, linoleate
18:3	C18:3n-3, α-linolenic acid, linolenate
^{2}H	deuterium
ACC	acetyl CoA carboxylase
ADA	American Diabetes Association
AHA	American Heart Association
apo	apoprotein, apolipoprotein
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
ATP	adenosine triphosphate
AUC	area under the curve
CDA	Canadian Diabetes Association
ChREBP	carbohydrate response element binding protein
ChORE	carbohydrate response element
CoA	coenzyme A
CPT-I	carnitine palmitoyltransferase I
CVD	cardiovascular disease
DGAT	diacylglycerol acyltransferase
DNA	deoxyribonucleic acid
DNFAn	net amount of de novo fatty acid (fractional synthetic rate as a %)
DNFAr	relative amount of de novo fatty acid
EDTA	ethylene diamine tetraacetic acid
FA	fatty acid
FABP	fatty acid binding protein

FAME	fatty acid methyl ester
FAS	fatty acid synthase
FSR	fractional synthetic rate
GPAT	glycerol-3-phosphate acyltransferase
GLC or GC	gas liquid chromatograph
HDL	high density lipoprotein
HF	higher fat intake
HOMA-IR	homeostasis model assessment of insulin resistance
IDL	intermediate density lipoprotein
kcal	kilocalories
LDL	low density lipoprotein
LF	lower fat intake
LPL	lipoprotein lipase
LXR	liver x receptor
MIDA	mass isotopomer distribution analysis
mRNA	messenger ribonucleic acid
MTP	microsomal triglyceride transfer protein
MUFA	monounsaturated fatty acid
NADPH	reduced nicotinamide adenine dinucleotide phosphate
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acid
RNA	ribonucleic acid
RXR	retinoid x receptor
S14	Spot 14
SCD1	stearoyl Coenzyme A desaturase 1
SFA	saturated fatty acid
SRE	sterol regulatory element
SREBP	sterol regulatory element binding protein
T3	triiodothyronine
TG	triglyceride, triacylglycerol
VLDL	very low density lipoprotein

CHAPTER 1

THESIS INTRODUCTION

High serum triglyceride levels frequently characterize the dyslipidemia that accompanies diabetic and insulin resistant states. Hypertriglyceridemia is a risk factor for cardiovascular disease, the leading cause of morbidity and mortality in diabetes (Sprecher, 1998). Diet has the potential to impact serum triglyceride levels by influencing lipogenesis and lipoprotein metabolism. However, the optimal macronutrient intake for prevention and treatment of hypertriglyceridemia in insulin resistant states is controversial. Dietary recommendations for individuals with diabetes have traditionally been focused on reducing cardiovascular disease via LDL cholesterol and weight loss strategies, rather than on ameliorating triglyceride metabolism. As a result, high carbohydrate/low fat diets have been prescribed and these diets have also been implicated in the etiology of hypertriglyceridemia. The phenomenon of "carbohydrate-induced hypertriglyceridemia," where low fat diets result in high levels of fat in the blood, is counterintuitive and has long been a challenge for clinicians and scientists to understand. The most apparent contributing mechanism would be the conversion of excess carbohydrate to fat, that is, *de novo* lipogenesis. Recent methodology has allowed investigation into the mechanisms underlying perturbations in plasma triglycerides in humans in vivo. Reliable quantification of de novo lipogenesis by use of stable isotopic procedures could contribute to further understanding of the etiology of hypertriglyceridemia, perhaps resulting in re-examination of the recommendation to reduce fat and increase carbohydrate intake.

1.1 LIPID METABOLISM

Lipids are major nutrients distinguished by their solubility in organic solvents. Roles of lipids include cortico-steroid hormones, energy sources, fat-soluble vitamins, as well as performing structural and functional duties within the cell. Lipids act as precursors of important second messengers (prostaglandins, prostacyclins, and leukotrienes) and function as structural constituents in cellular membranes, influencing the activities of membrane-linked molecules such as receptors, enzymes, and transporters (Clandinin *et al*, 1991;Clandinin *et al*, 1993;Clandinin *et al*, 1994;Fernstrom, 2000).

The most abundant form of dietary lipid is triacylglycerol, commonly referred to as 'triglyceride' or 'fat'. Dietary fat affects gene expression, resulting in changes in metabolism, cell differentiation, and growth. Adaptive changes to variations in dietary fat amount or composition have genomic effects, and this may greatly influence human health and chronic disease progression (Jump & Clarke, 1999).

There are 2 main sources of triglyceride (TG) in the body; the diet and the liver (Sprecher, 1998), respectively known as exogenous and endogenous sources. The major function of TG is to provide cells with energy, contributing approximately 9 kilocalories per gram, compared with the 4 kilocalories per gram provided by protein and carbohydrate. Due to the hydrophobicity of TG, unique digestion and transport mechanisms are required in the aqueous environment of the body. Hence, dietary TG is packaged into chylomicrons and liver TG is transported within very-low density lipoproteins (VLDL).

1.1.1 DIGESTION AND ABSORPTION

Digestion of dietary TG begins in the stomach by mechanical separation and then partial hydrolysis by salivary lipase. It continues in the lumen of the small intestine, where emulsification occurs. The peristaltic movement of the duodenum and the action of bile salts aid in breaking the fat globules into smaller micelles, which exposes surface area to pancreatic triglyceride lipase. The binding of colipase to pancreatic triglyceride lipase is necessary in reducing bile salt inhibition and restoring activity of the enzyme (Lowe, 1997).

Micelles contain products of TG digestion; 2-monoacylglycerols and fatty acids. These are water soluble and able to pass through the unstirred intestinal water layer. Interaction occurs with the brush border of the distal duodenum and jejunum, whereby the lipid constituents of the micelle dissociate from the bile salts and diffuse into the enterocytes (Groff *et al.*, 1995). Measurements of fecal fat excretion have indicated that at least 95% of fat ingested is absorbed, but this may vary depending on the positional distribution of a fatty acid, chain length and saturation (Mu & Hoy, 2004).

The diffusion of long chain fatty acids (FAs) was once thought to be passive, but evidence has indicated the involvement of fatty acid binding proteins (FABPs). It has been suggested that FABPs may play a role in protecting against the adverse effects of high fatty acid concentrations, as well as assist in the transfer of long-chain fatty acids between membranes and fatty acid metabolism sites (Hsu & Storch, 1996;Storch & Thumser, 2000). Reconstruction of TG from dietary FA and monoglycerides occurs by re-esterification in the smooth endoplasmic reticulum of the intestinal mucosal cells.

Short and medium chain free fatty acids are not re-esterified and pass directly into portal circulation. However, all other exogenously derived TG and lipids are packaged into chylomicrons, large lipoprotein particles, for transport to the rest of the body. Chylomicrons are not released into the portal system, but are secreted into the lymphatic system for gradual release into the bloodstream. This prevents large changes in postprandial serum lipid content, peaking at approximately one-half to three hours following a meal, and returning to normal again within five to six hours under normal circumstances. Very low-density lipoproteins are also produced by the small intestine, however these are relatively few and are distinct from liver VLDL (Black, 1995).

1.1.2 FATTY ACID TRANSPORT

Non-esterified fatty acids are transported in circulation as free FAs complexed to albumin. These are derived from adipose tissue TG lipolysis, TG-rich lipoprotein lipolysis (chylomicrons, VLDL) or absorbed FAs (Sparks & Sparks, 1985). However, most lipids are transported in the bloodstream within lipoprotein particles, stabilizing them in the aqueous environment.

Lipoproteins consist of a hydrophobic core (i.e. TG, cholesterol esters) and an outer layer of phospholipids and apoproteins. Chylomicrons formed from exogenous sources are the largest and least dense lipoprotein particles, with a Svedberg unit (S_f) of >400. These particles contain one molecule of apoprotein (apo) B-48 and function to transport dietary TG to the body following a meal. Blood levels of chylomicrons are very low in the fasting state (Parks & Hellerstein, 2000).

The apolipoproteins (apo) perform essential functions in the secretion, metabolism, and receptor-mediated uptake of these particles (Black, 1995). For example, apoC-II acts a cofactor for lipoprotein lipase (LPL), hydrolyzing the TG from VLDL for release to cells for oxidation (muscle) or storage (adipose). A chylomicron remnant remains, which is smaller and contains relatively more cholesterol. These are cleared from the bloodstream when hepatic cell receptors for apoE take up and catabolize the exogenous lipids within the remnants (Groff *et al*, 1995).

The liver re-esterifies monoglycerides and FAs from both exogenous and endogenous sources into TG and repackages it along with other lipids in VLDL. Once secreted into circulation by the liver, LPL acts to hydrolyze the TG from the VLDL for uptake by the cells. The result is a temporary intermediate density lipoprotein (IDL). IDL become depleted in TG and are taken up by the liver or converted into low-density lipoprotein. Low-density lipoprotein (LDL) is rich in cholesterol, transporting it to tissues for uses such as membrane structure or steroid hormone synthesis. High-density lipoprotein (HDL) is the smallest and densest lipoprotein and consists mostly of phospholipids and apoprotein with relatively little TG and cholesterol. It functions to remove unesterified cholesterol from cells and other lipoproteins for return to the liver (Groff *et al*, 1995).

Fatty acids lipolyzed from lipoproteins and plasma free fatty acids need to enter cells in order to be available for metabolism. Fatty acid transport across membranes occurs in three steps. First the monomeric unbound FA adsorbs to the outer plasma membrane. Transmembrane movement occurs next, followed by desorption of the FA for intracellular utilization (Hamilton, 1998). It has been unclear as to whether some of these steps occur solely by passive diffusion or if the process is protein-facilitated. Shorter chain fatty acids may diffuse rapidly and spontaneously, whereas the transport of larger more hydrophobic lipids or very long chain fatty acids may be facilitated by association with albumin, lipocalins and fatty acid-binding proteins (Hamilton, 1998). Integral membrane proteins thought to be involved in FA uptake include CD36, fatty acid transport protein (very long chain acyl-CoA synthetases) and caveolin-1. FABPs may also be important for transport of FAs within cells to sites of activation to acyl-CoAs, which are thereafter carried within the cell by acyl-CoA binding protein. The rate of fatty acid entry into a cell is thought to be regulated by binding to intracellular proteins, or due to metabolism by way of synthetic or degradative pathways (Mashek & Coleman, 2006).

1.2. PLASMA TRIGLYCERIDE LEVELS

1.2.1 TRIGLYCERIDE-RICH LIPOPROTEINS

Plasma triglycerides are made up primarily of TG from chylomicrons, VLDL and IDL and are collectively referred to as triglyceride-rich lipoproteins (TRLs). As previously mentioned, TRLs circulate in the bloodstream until depleted of their TG load and are taken up by receptor-mediated processes into the liver. The balance between rate of production and secretion of TRLs and rate of clearance determines plasma TG concentration.

VLDL Subfractions

Although VLDL are separated into two main subfractions according to density (VLDL1: Sf 60-400; VLDL2: Sf 20-60), most studies refer to both as VLDL. It should also be noted that chylomicron remnants, if present, may also appear in the VLDL fraction as their density is similar to VLDL1. Research has indicated that VLDL1 shows the greatest increase in TG after a meal (Fisher *et al*, 1993) and has the shortest residence time of the two subfractions. Although a portion of VLDL1 is hydrolyzed and converted to VLDL2, the liver appears to secrete VLDL into both density ranges. When each VLDL species originates directly from the liver, it is thought that their regulation is independent and each may be targeted for different fates (Packard & Shepherd, 1997). For instance, it seems that <10% of VLDL1 is converted to LDL, whereas >50% of VLDL ends up as LDL.

VLDL versus Chylomicrons

The majority of plasma TG at any point in time is carried in VLDL, particularly during fasting when chylomicrons are mostly absent from plasma (Schaefer *et al*, 1978). The amount of chylomicron-TG in plasma tends to be much less than that in VLDL, as the contribution of chylomicron-TG to total TG may only reach as high as 20% following a meal (Schneeman *et al*, 1993). This was shown to be true even following a 1g/kg fat load, where even though the greatest increase in postprandial TG occured in chylomicrons, the total amount of TG carried by TRLs in the postprandial period was higher in VLDL. Average VLDL-TG amount was not exceeded by chylomicron TG, however individual values varied greatly for the latter, ranging from 6-56% (Cohn *et al*, 1993).

During the postprandial state there is a competition between VLDL and chylomicrons for lipolysis and removal. Chylomicron-TG seems to be hydrolyzed much more efficiently (Grundy & Mok, 1976) with chylomicron lipolysis being preferred by LPL (Potts *et al*, 1991) shortening the residence time in plasma. Although in some hypertriglyceridemic subjects there may still be some detectable apoB-48 (i.e. chylomicron remnants) present at 9 hours post-meal (Schneeman *et al*, 1993).

The turnover rate of the various lipoproteins seems to vary between individuals. In normal subjects, some studies have estimated VLDL residence time in plasma of up to one hour and just over an hour for hypertriglyceridemic subjects (Barrett *et al*, 1991). Chylomicrons may exist from the point of secretion by the intestine to uptake by the liver for anywhere from 15 to 30 minutes. Indeed, half life has been calculated at as little as 4.5 min in normolipidemic subjects and up to 23 minutes in hyperlipidemic subjects (Grundy & Mok, 1976).

1.2.2 VLDL METABOLISM

Triglyceride is synthesized and secreted by the liver into VLDL particles and reaches circulation via hepatic sinusoids. These lipoproteins function to provide a constant supply of TG to peripheral tissues for storage or oxidation, as well as cholesterol transport via degradation to LDL.

VLDL Apolipoproteins

The apolipoproteins specific to VLDL determine its role as a lipoprotein particle. Very low density lipoproteins contain apoB, apoC-I, apoC-II, apoC-III, and apoE in varying amounts (Sparks & Sparks, 1985).

Apolipoprotein B-100 (apoB-100) is the largest protein component of VLDL, and does not exchange between lipoproteins. However, it is also present in LDL due to the conversion of VLDL to LDL. ApoB-100 is synthesized in the liver and is critical to the initial assembly and secretion of VLDL (Ginsberg, 1998). Translocation into the lumen of the endoplasmic reticulum is dependent on a supply of lipid; otherwise it remains membrane-bound and is degraded (White *et al*, 1998). There follows a competition for apoB-100 between assembly into VLDL and degradation. As apoB-100 is passed through the secretory pathway undegraded, it acquires TG and other components (cholesteryl ester, phospholipid) and is secreted as VLDL (White *et al*, 1998).

ApoC-I may function to inhibit hepatic uptake of chylomicron and VLDL remnants. ApoC-II is mainly associated with VLDL in the fasting state and is activated by LPL to facilitate TG hydrolysis (Ginsberg, 1998). Its absence results in severe hypertriglyceridemia with chylomicronemia. ApoC-III is a major component of VLDL, and inhibits both LPL action and hepatic uptake of chylomicron and VLDL remnants (Ginsberg, 1998).

ApoE is synthesized in the liver and has a critical role in the removal of remnant lipoproteins from plasma by interacting with receptors in the liver (Ginsberg, 1998;Mahley & Ji, 1999). It was proposed (Davignon *et al*, 1988) and later corroborated (Millar *et al*, 2001) that the content of apoE on VLDL may be a major determinant in whether VLDL are cleared from plasma or further metabolized to LDL.

Synthesis and Secretion

VLDL synthesis is affected by factors such as genetics, diet and hormones, by influencing either the number or size, and lipid content of particles secreted (Sparks & Sparks, 1985). The process of VLDL synthesis and secretion continues to evolve as research attempts to elucidate mechanisms of regulation. Several processes must come together to coordinate this process including the availability of triglyceride, the synthesis of ApoB-100, and the influence of hormones (insulin) and enzymes (microsomal triglyceride transfer protein).

Briefly, fatty acids enter the hepatocyte, are oxidized to ketone bodies or esterified to cytosolic TG, and then lipolyzed and re-esterified as the major source of TG for VLDL assembly (Gibbons & Wiggins, 1995). Evidence suggests that there are specific TG pools, and that FAs of endogenous and exogenous origin are compartmentalized in these pools and then mobilized and targeted toward specific pathways (Coleman *et al*, 2000). It is estimated that about 60%–70% of the FA esterified into VLDL-TG is derived from lipolysis of a cytosolic TG pool (Coleman *et al*, 2000). If lipolysis is suppressed, VLDL secretion seems to be inhibited (Coleman *et al*, 2000). Therefore, a site for regulation of hepatic VLDL output may be the lipolysis of cytosolic TG and transfer of FAs into the ER lumen (Gibbons & Wiggins, 1995). Any excess FA lipolyzed in the liver but not targeted for secretion is either oxidized or returns to the cytosolic TG pool (Coleman *et al*, 2000).

It is known that three factors are absolutely required for VLDL synthesis and secretion to occur: lipid, apoB and microsomal triglyceride transfer protein (MTP). First, apoB-100 is synthesized and after translocation through the ER membrane, the protein is either targeted for secretion or degradation. The successful secretion of apoB is partially dependent on the availability of and subsequent association with core lipid (Boren *et al*, 1991) and degradation occurs if lipid fails to associate with apoB. ApoB will be degraded if lipid is absent, if it is taken up before this step, or if there is a deficiency of MTP (Ginsberg, 1998). In vitro oleate treatment of HepG2 cells stimulates apoB secretion and reduces degradation by increasing triglyceride synthesis, thereby accelerating translocation (Sakata *et al*, 1993). It has been suggested that in vitro the availability of lipid may be rate limiting, whereas when lipid is in excess in vivo, apoB processing may be the limiting factor (Wang *et al*, 1997). Control of VLDL synthesis and secretion is influenced by nutritional and hormonal state, through several transcription factors. These include SREBP-1c (via lipogenesis) and PPARα (via MTP and FABP1) (Blasiole *et al*, 2007).

1.2.3 LIPOGENESIS

Lipogenesis is the term used to collectively describe the synthesis of TG and/or the constituent FAs. Lipogenesis occurs in the intestinal mucosal cells, the liver, adipose tissue and mammary glands during lactation (Vernon *et al*, 1999). Endogenous synthesis of FAs and TG in the liver takes place when total energy intake exceeds energy expenditure (Sul & Wang, 1998), particularly with diets low in fat and high in carbohydrate. Whereas the exogenous cycle is initiated at the intestine and involves the resynthesis and transport of TG into the circulation, the liver initiates the endogenous portion of TG metabolism. The liver has a central clearing role, taking up and esterifying circulating fatty acids from plasma, and synthesizing FAs *de novo* from the catabolism of carbohydrates and amino acids (Groff *et al*, 1995). When excess glucose reaches the liver, particularly when liver glycogen stores are saturated, a lipogenic response occurs (Towle *et al*, 1997;Acheson *et al*, 1988). The excess energy is eventually stored as TG in adipose tissue (Sul & Wang, 1998).

The expression of genes encoding both glycolytic and lipogenic enzymes can be inhibited or upregulated within hours of a single meal (Jump *et al*, 1994). This short term regulation of lipogenesis involves rapid activation of critical enzymes through hormonal and metabolic influences on posttranslational modifications and allosteric mechanisms (Towle *et al*, 1997). Long-term regulation by nutritional and hormonal factors involves adjustments at the transcriptional level ultimately determining enzyme concentrations. Research in rodents has shown that fasting then refeeding with a high carbohydrate/low fat diet will result in an induction process specifically increasing the concentrations of enzymes involved in lipogenesis (Sul & Wang, 1998).

Although both FA synthesis and TG synthesis are part of lipogenesis, both should be considered two distinct processes in fat metabolism. *De novo* fatty acid synthesis refers to the biosynthesis of FAs from non-lipid precursors, that is, carbohydrate and amino acids. TG synthesis refers to the assembly of TG from acyl CoA precursors, originating from either exogenous (diet) or endogenous (*de novo* FA synthesis) sources. *De novo* lipogenesis is the synthesis of TG from FAs of endogenous origin (*de novo*) or via chain elongation. Of course, TG synthesis usually includes a number of sources, making most TG molecules a mixture of exogenous and endogenous source FAs.

Fatty Acid Synthesis

Reactions of *de novo* FA synthesis (long chain fatty acid biosynthesis) are catalyzed in two main steps by the multi-enzyme systems; acetyl-coenzyme A carboxylase (ACC) and fatty acid synthase (FAS) in the cytoplasm (Wakil *et al*, 1983;Sul & Wang, 1998). The lipogenic enzymes ACC and FAS are under long term control at transcriptional and translational levels, where diet and insulin are important in regulating enzyme synthesis rates (Wakil *et al*, 1983). FAS, ACC and malic enzyme are believed to be the major rate-limiting enzymes in lipogenesis. However, since glucose metabolic products are also involved in de novo synthesis, the enzymes glucokinase, pyruvate kinase and glucose-6-phospate dehydrogenase could also be considered rate-limiting (Hillgartner *et al*, 1995). This is not the case for fructose, as the metabolism of this simple carbohydrate bypasses the rate-limiting enzymes involved in glucose metabolism.

After glucose is converted to pyruvate and then acetyl CoA, it must be transported into the cytosol in the form of citrate. Citrate is then converted back to acetyl CoA, a reaction that reproduces oxaloacetate. This is converted to malate, which in turn is converted to pyruvate via malic enzyme, producing NADPH. Pyruvate is transported back into the mitochondria to participate in the process again (Herzberg, 1991). This initiates the first critical step in FA synthesis. ACC, which requires biotin as a prosthetic group for the carboxylation reaction, converts acetyl-CoA to malonyl-CoA. The malonyl-CoA produced is an essential substrate for FAS, but is also required by chain elongation systems to produce important 20 carbon FAs involved in the synthesis of other lipids, such as eicosanoids, sphingolipids and glycolipids (Poulos, 1995). Increased levels of malonyl-CoA also inhibits carnitine palmitoyl-CoA transferase-I (CPT-I), a key regulator of fatty acid oxidation. If rising levels of malonyl CoA did not inhibit CPT-I, a futile cycle would occur allowing the acyl-CoA to react with carnitine and convert back to acetyl-CoA through ß-oxidation. Conversely, when malonyl CoA inhibits CPT-I, the long chain acyl-CoA formed can react with glycerol phosphate to form TG. In this way, malonyl CoA becomes an important coordinator of hepatic fatty acid synthesis and β-oxidation rates (McGarry, 2002).

The conversion of acetyl-CoA and malonyl-CoA to palmitate, catalyzed by FAS and requiring NADPH, is the second critical step in FA synthesis (Wakil *et al*, 1983). This conversion requires the cyclical involvement of seven different enzymes, five of which perform eight times to carry out 37 repetitive reactions. This sequential chain elongation results in one molecule of the saturated FA palmitate (16:0) from the 30-acyl intermediates involved (Wakil *et al*, 1983). Further elongation reactions produce longer chain FAs such as stearate (18:0).

Once *de novo* fatty acid synthesis has produced palmitate and stearate, desaturation reactions result in conversion to the corresponding monounsaturated fatty acids, such as oleic acid ($18{:}\Delta^9$ or 18:1). Stearoyl-CoA desaturase-1 (SCD1) is a Δ-9 desaturase known to catalyze the conversion of 16:0-CoA to 16:1-CoA and 18:0-CoA to 18:1-CoA. Although humans can introduce double bonds to the fatty acid chain starting at the Δ-9 position, the enzymes required for further desaturation reactions (Δ^{12} and Δ^{15} desaturases) are lacking (Lehner & Kuksis, 1996). Fatty acids of the n-3 and n-6 families must therefore be acquired from dietary sources, rendering linoleic acid ($18{:}\Delta^{9,12}$ or 18:2n-6) and α-linolenic acid ($18{:}\Delta^{9,12,15}$ or 18:3n-3) essential. The enzymes involved in the metabolism of these essential FAs to longer chain polyunsaturated FAs are shared between the n-3 and n-6 FAs. Consequently there exists a competition in the formation of eicosapentaenoic acid ($20{:}\Delta^{5,8,11,14,17}$ or 20:5n-3) and docosahexaenoic acid ($22{:}\Delta^{4,7,10,13,16,19}$ or 22:6n-3), with arachidonic acid ($20{:}\Delta^{5,8,11,14}$ or 20:4n-6). An imbalance between these fatty acid families is thought to affect health over the long term.

Despite additional reactions synthesizing longer chain FAs, it has been shown that the FA preferentially formed by mammalian FAS is palmitate (Hudgins *et al*, 1996;Aarsland & Wolfe, 1998). Stearic acid and oleic acid are also incorporated into VLDL-TG, but to a much lesser extent, even during high carbohydrate feeding (Aarsland & Wolfe, 1998). This may be because these FAs are preferentially oxidized (18:1) or incorporated into phospholipids (18:0)(Bruce & Salter, 1996). The stimulation of lipogenesis by high carbohydrate intake also creates an increase in saturated fatty acid and decrease in linoleic acid content of VLDL-TG (Hudgins, 2000), plasma phospholipids and cholesterol esters (King *et al*, 2006). It is known that the amount of de novo FA synthesis is highly variable between animals and humans and between individuals. However it is not known whether the FAs produced de novo also vary considerably between species, individuals, diets or disease states, and what the consequences of varying rates may be.

Triglyceride Synthesis

The major pathway involved in exogenous TG synthesis is the monoglyceride pathway. The enzymes acyl-CoA synthetase, monoglyceride transacylase and diglyceride transacylase work together in what is known as the triglyceride synthetase complex. FAs form acyl-CoA in the presence of CoA and acyl-CoA synthetase. The acyl-CoA is then used in the reacylation of monoglycerides to diglycerides and TG (Tso, 1985).

The α-glycerophosphate pathway (also referred to as the phosphatidic acid pathway or the glycerol-3-phosphate pathway) is the *de novo* route of TG formation. Acyl-CoA is formed from FAs and CoA, not unlike in the monoglyceride pathway. However, as in the enterocyte during fasting, the lack of monoglyceride from diet results in the use of glycerol. Glycerol kinase acts to convert it to L-α-glycerophosphate, which is converted to phosphatidic acid and then to diglyceride for acylation to TG (Tso, 1985). These last two conversions, catalyzed by phosphatidate phosphohydrolase and diacylglycerol acyltransferase respectively, appear to be the rate-limiting steps (Herzberg, 1991).

There are 3 sources of FAs for TG synthesis in the liver. As previously mentioned, de novo lipogenesis provides FAs synthesized from carbohydrate or amino acid sources. Circulating free FAs derived from adipose tissue TG lipolysis are also a source of FAs and considered to be a major driver of TG synthesis and secretion in VLDL. Finally, dietary FAs can arrive at the liver either by TG-rich lipoprotein lipolysis 'spillover' into the plasma free FA pool or through uptake of TG-rich lipoprotein remnants into the liver. Hepatic cytosolic TG stores can also be utilized for VLDL-TG synthesis, however this source of FAs ultimately originated from one of the other 3 sources. The proportion from each source contributing to the FAs in VLDL-TG is an area of currently being studied and seems to vary depending on the metabolic state of the individual (i.e. insulin sensitivity, fed vs. fasted).

According to human studies using stable isotopes, the utilization of FAs from hepatic TG stores results in a delay in secretion of fatty acids from the other sources for secretion into VLDL. Labeled FAs from a meal may appear in VLDL-TG up to 2 hours following that meal or may be secreted into VLDL as late as 12 hours later (Vedala *et al*, 2006). This suggests that functionally separate pools of TG may exist and that some of the secreted VLDL–TG may originate from cytosolic TAG rather than from new synthesis from exogenous fatty acid (Coleman & Lee, 2004).

1.3 FACTORS INFLUENCING PLASMA TRIGLYCERIDES

Changes in dietary carbohydrate and fat composition affect metabolic processes directly and indirectly in the short, intermediate and long term. Diet affects the amount and type of fuel uptake by the target tissue and metabolic intermediates affect enzymes in pathways and subsequent hormonal responses (Leahy *et al*, 1999). In turn, there are alterations in the concentration of proteins for specific pathways such as gene transcription, RNA transport and processing, some protein synthesis and post-translational modification (Sul & Wang, 1998). The expression of genes for lipogenic enzymes responds to a variety of factors including FAs, glucose, insulin, thyroid hormone, catabolic hormones and leptin.

1.3.1 FATTY ACIDS

Dietary Fat

Lipogenesis seems to be inhibited if greater than 30% of energy from the diet is derived from fat (Herzberg, 1991;Jeffcoat *et al*, 1979). However, both dietary fat amount and composition are important in the regulation of lipogenesis. It has been shown that lipogenesis can be suppressed in rats when diets are supplemented with linoleic acid, versus the consumption of an unsupplemented diet or one supplemented with beef tallow (Jeffcoat *et al*, 1979). In mice, when the ratio of polyunsaturated to saturated fatty acids is high, there results a suppression of FAS, malic enzyme and ACC gene expression and enzyme activities (Cheema & Clandinin, 1996). Animal and human studies (*in vitro* and *in vivo*) have demonstrated that polyunsaturated FAs, particularly n-3 FAs, decrease serum TG and VLDL levels (Sebokova *et al*, 1996;van Vlijmen *et al*, 1998;Brown *et al*, 1999). This reduction in VLDL has been attributed to inhibition of de novo FA synthesis, hepatic cellular TG synthesis and VLDL secretion (Brown *et al*, 1999), as well as increased VLDL apoB fractional catabolic rate (van Vlijmen *et al*, 1998).

The molecular mechanisms by which polyunsaturated FAs reduce lipogenesis are currently being elucidated. It seems that these essential FAs act as ligands for many nuclear receptors such as PPARs, however, this may not be the only mechanism by which polyunsaturated FAs function (Clarke & Jump, 1996). Similarly to other lipogenic enzymes, stearoyl-CoA desaturase-1 (SCD1) activity decreases with starvation/insulin deficiency and increases with high saturated fat diets, whereas polyunsaturated FAs suppress SCD-1 mRNA. Interestingly, there is some evidence that the activity of a desaturase enzyme may affect the total synthesis of TG (Jeffcoat, 2007).

Non-esterifed Fatty Acids

Although previously most VLDL-TG was thought to derive from intracellular lipolysis of TG, evidence suggests that plasma non-esterified or free fatty acids not only stimulate VLDL synthesis and secretion but may be the main substrate for VLDL-TG (Byrne *et al*, 1991). Circulating free FAs, derived mostly from adipose tissue TG lipolysis or TG-rich lipoprotein lipolysis (Sparks & Sparks, 1985), have an effect on hepatic processes. Research suggests that free FA flux into the liver and the rate of re-esterification to TG are major determinants for TG synthesis (Malmstrom *et al*, 1997b). *In vitro* studies have shown that free FAs stimulate TG and VLDL secretion (human hepatoma cell culture medium) (Dashti & Wolfbauer, 1987;Byrne *et al*, 1991). The activity of the key enzymes in TG production, such as phosphatidate phosphohydrolase and diacylglycerol acyltransferase, increase in the presence of non-esterified FAs (Byrne *et al*, 1991).

Normally, during fasting, the quantitative substrate source that seems to drive VLDL synthesis is plasma free FAs. According to isotope tracer studies, free FAs from adipose tissue lipolysis can amount to approximately 77% of VLDL-TG FAs, whereas about 4% are derived from de novo lipogenesis. During the fed state, these sources seem to switch somewhat, due to the presence of high insulin. Hence, as free FA lipolysis from adipose tissue decreases, so does the contribution to VLDL-TG. Following a meal, the contribution of plasma free FAs may decrease to 44%, whereas de novo lipogenesis doubles to 8% of FAs in VLDL. Other sources make up the remainder of the change postprandially, including 22% dietary FAs from hepatic uptake of chylomicron remnant TG and FA spillover from lipoprotein lipolysis into the plasma free FA pool (Barrows & Parks, 2006). Chronic hyperinsulinemia may influence the sources of VLDL-TG FAs. Research examining FAs from the adipose tissue of obese subjects showed continual release and free FA mobilization even under conditions promoting suppression. This was attributed to impaired inhibition of hormone sensitive lipase and low retention of FAs for reesterification within the adipose tissue (Coppack *et al*, 1992).

Conceivably, the sources for hepatic VLDL-TG synthesis will influence the composition of TG stored in the liver and hence the intracellular non-esterified FA pool. The composition of the intracellular non-esterified FA pool is affected by FA entry and exit (i.e. FA transporters, synthesis, oxidation). Fatty acid structure may be an important determinant of many of these functions. Also, the composition of this pool may be affected by drugs, disease state and genetic background, which in turn affects lipid metabolism and FA regulated nuclear receptor activity (Pawar & Jump, 2003). For instance, FAs with a chain length of <14 or >20 carbons are poor ligands for PPARs (Xu *et al*, 1999) and 18- and 20-carbon *n*-6 PUFA, but not *n*-3-PUFA, are preferred substrates for cyclooxygenase- and lipoxygenase-dependent eicosanoid synthesis (Jump, 2002). Studies by Pawar and Jump using rat primary hepatocytes indicated that several factors may control hepatic levels of PPAR ligands including lipogenic and peroxisomal beta-oxidation enzyme activities, as influenced by non-esterified FA composition (Pawar & Jump, 2003).

1.3.2 CARBOHYDRATE

Carbohydrates are known to be lipogenic, particularly the overconsumption of simple sugars. As blood glucose concentration increases postprandially, there is a subsequent increase in the rate of glucose entry into the liver and enzymes are directed away from glucose synthesis and glycogen breakdown and toward glycogen synthesis (Leahy *et al*, 1999). Once glycogen stores are saturated, expression of the genes for FAS and ACC are increased due to the metabolism of excess glucose and stimulation of transcription (Girard *et al*, 1997). This stimulation of transcription seems to be partially accomplished by nuclear response elements (see 1.3.4 Gene-Nutrient Interactions: Transcription Factors).

Changing glucose level also signals the secretion of hormones (Sul & Wang, 1998). As previously mentioned, these hormones, particularly insulin and glucagon, can affect regulation in both the short and long term. In the short term, the rise in insulin concentration that follows carbohydrate consumption and high blood glucose levels causes an induction of the enzymes involved in FA and TG synthesis (Sul & Wang, 1998).

Fructose consumption was originally recommended to individuals attempting to achieve better blood glucose control. However, high fructose intake tends to increase lipogenesis in both normal and insulin resistant states. It does this in part by affecting gene expression by glucose, indirectly influencing glucose metabolism by stimulating glucokinase activity. This is thought to be carried out via a signaling metabolite such as glucose-6-phosphate (Girard *et al*, 1997). Fructose also bypasses the rate-limiting step in glycolysis and therefore proceeds directly to the pathway of lipogenesis via glyceraldehyde-3-phosphate.

Diets rich in glucose, fructose and sucrose provide substrates for and stimulate genes encoding enzymes in the glycolytic and lipogenic pathways, and in conjunction with stimulation of insulin secretion, result in an increase in lipogenic capacity (Girard *et al*, 1997). Carbohydrate-induced hypertriglyceridemia, associated with high rates of de novo lipogenesis, is a persistent effect of high carbohydrate diets in humans, even when consuming whole food, high fibre diets (Parks *et al*, 1999). Adding fibre to a moderately high carbohydrate diet (Letexier *et al*, 2003), or consuming a mixed carbohydrate diet that replaces sugar with starch and fibre may reduce both lipogenesis and plasma TG levels (Hudgins *et al*, 1998).

Glucose metabolism may influence other steps in VLDL, such as the lipid addition step in VLDL assembly. *In vitro*, glucose increases the synthesis of apoB, thereby stimulating hepatic TG synthesis, turnover and output of VLDL. Interference in glucose phosphorylation prevents these effects (Brown *et al*, 1999).

1.3.3 HORMONES

Insulin

Together, elevated circulating levels of glucose and insulin induce enzymes involved in FA and TG synthesis (Sul *et al*, 2000). This occurs indirectly via insulin stimulation of glucose transport and metabolism, however insulin has important effects on TG synthesis that are in addition to stimulating glucose uptake through direct stimulation of these enzymes (Girard *et al*, 1997). In diabetic mice, insulin administration causes a rapid increase in mRNA and transcription rate of the FAS gene (Paulauskis & Sul, 1989). Insulin likely binds to cell surface receptors, which results in regulation of FAS transcription through activation of signaling pathways (Sul *et al*, 2000). Insulin may also stimulate rapid increases in malic enzyme gene expression, possibly by direct signaling to the hepatic nucleus via insulin receptor translocation (Gletsu *et al*, 1999). Alternatively, in periods of fasting when glucagon is elevated, intracellular cAMP levels increase and the activities of enzymes in FA and TG synthesis are suppressed (Sul & Wang, 1998;Sul *et al*, 2000). Insulin promotes lipogenesis in liver and adipose tissue largely by increasing the expression of SREBP-1c.

Studies involving insulin and its effect on VLDL-TG production and secretion have been conflicting. It is now accepted that insulin tends to be hypotriglyceridemic under normal conditions (Malmstrom *et al*, 1997a). Insulin decreases plasma TG by inhibiting adipose tissue lipolysis, decreasing non-esterified free FA flux into plasma and activating adipose tissue LPL (Byrne *et al*, 1991;Parks & Hellerstein, 2000). Since the presence of free FAs normally causes an increase in the lipogenic activities of the liver, reducing this flux from adipose tissue was initially thought to be the main mechanism by which insulin acted. However, it has been shown *in vitro* using HepG2 cells, that insulin inhibits TG and TG-rich lipoprotein secretion even in the presence of free FAs, albeit to a lesser extent (Dashti & Wolfbauer, 1987;Byrne *et al*, 1991). It is possible that insulin has deleterious effects on TG metabolism only in conditions of hyperinsulinemia and/or insulin resistance. This could be because resistance to insulin action may not occur in all tissues simultaneously. For instance, some response elements in the liver may be insulin responsive, while those in peripheral tissues remain resistant.

Evidence suggests that insulin may suppress VLDL apoB production (Lewis *et al*, 1995;Malmstrom *et al*, 1998). Specifically, liver apoB production of the different VLDL subfractions may be regulated independently, with hyperinsulinemia suppressing mostly VLDL1 apoB production (Malmstrom *et al*, 1997b). The liver may secrete larger VLDL as hepatic TG synthesis increases. These TG-rich particles are less efficiently converted to LDL and consequently plasma TG levels increase (Caslake *et al*, 1992). By decreasing the production of large TG-rich VLDL1 particles in favour of smaller and denser VLDL2 particles, insulin thereby suppresses the total production rate of VLDL apoB (Blasiole *et al*, 2007).

Leptin

Leptin is a hormone produced by the OB gene and has a variety of functions related to body energy homeostasis, including influencing glucose and lipid metabolism (reviewed by Baile *et al*, 2000). Leptin levels increase following a meal and begin to decrease several hours after a meal. It is thought that leptin may affect lipogenesis through insulin-dependent and insulin-independent mechanisms (Lee *et al*, 2000). The hormone also alters synthesis and uptake of FAs (Baile *et al*, 2000). Research in rats has shown that leptin suppresses expression of lipogenic genes (i.e. ACC and FAS) (Zhou *et al*, 1998;Fukuda *et al*, 1999). It seems that leptin receptor-defective ZDF rats lose their ability to regulate lipogenesis during adaptation to changes in diet. This may include dysregulation of chain elongation and desaturation of nonessential fatty acids in response to a change in the amount of fat and carbohydrate in the diet (Lee *et al*, 2000).

Thyroid Hormone

The thyroid hormone triiodothyronine (T3) appears to be lipogenic due to its stimulatory effect on expression of FAS (Xiong *et al*, 1998), ATP-citrate lyase (Brown *et al*, 1997), SCD (Joshi & Aranda, 1979) and the nuclear protein Spot 14 (Liaw *et al*, 1983).

T3 may be synergistic with a high-carbohydrate diet, independent of insulin stimulation (Moustaid & Sul, 1991;Walker *et al*, 1996). Rat studies have demonstrated that Spot 14 is induced by thyroid hormone and dietary carbohydrate and is involved in the transduction of hormonal and dietary signals, resulting in the stimulation of hepatic lipogenesis (Kinlaw *et al*, 1995). Alternatively, diabetes, polyunsaturated FAs, fasting and glucagon seem to inhibit Spot 14 expression (Girard *et al*, 1997). Spot 14 may be involved in the pretranslational regulation of lipogenesis (Kinlaw *et al*, 1995), however, evidence in knockout mice indicates that it is probably only one of the pathways inducible by lipogenic conditions (Zhu *et al*, 2001). Using chick embryo hepatocytes, Zhang et al. (Zhang *et al*, 2003) were able to identify an interaction between the SREBP-1 and T3 receptor signaling pathways. T3 appears to increase the concentration of the mature, active form of SREBP-1 (see Transcription Factors) and this is one mechanism by which insulin, cAMP, and medium-chain FAs may regulate ACC transcription.

1.3.4 GENE-NUTRIENT INTERACTIONS

Genes manifest as inherent phenotypic characteristics but also interact with diet and hormones. This area of research has been attempting to explain the individual variation observed in plasma lipids, lipid metabolism, as well as the response of these factors to diet changes.

It has been suggested (Masson *et al*, 2003) that gene-diet interactions are difficult to study conclusively because sample sizes are usually too small to detect a difference, especially for rare alleles. Diet protocols in regards to nutrient intake, fasting vs. fed states, anthropometrics and demographics of subjects tend to vary greatly within and between studies. Most research also seems to study single gene effects but the lipid response to changes in diet is probably under polygenic control and could be interacting with other functional mutations. Therefore research examining the effect of a single gene may only show a relatively small effect.

Single Nucleotide Polymorphisms

Single nucleotide gene polymorphisms have been studied in relation to cardiovascular disease risk and in response to dietary changes. These include apoproteins E, B, A-IV and C-III, as well as the LDL receptor, microsomal transfer protein, fatty acid-binding protein, cholesteryl ester transfer protein, lipoprotein lipase and hepatic lipase. Several have been shown to explain some of the variation in plasma TG response to diet (i.e. apoE, intestinal FABP) (Vincent *et al*, 2002).

Hypertriglyceridemia is known to manifest from overt genetic abnormalities in lipoprotein physiology and/or metabolism. Specific polymorphisms of apoprotein genes can affect synthesis and clearance of VLDL particles. This was observed in the apo A-I/C-III/A-IV gene cluster in isolated hypertriglyceridemia and combined hyperlipidemia (Talmud & Humphries, 1997). Individuals may have a dysfunction in apoC-III production or an increase in the ratio of apoC-III to apoC-II resulting in hypertriglyceridemia (Ginsberg, 1998). Lipoprotein lipase and apoC-II deficiency both cause very high TG levels and have been defined as type I and type V hyperlipoproteinemia, respectively. Type III hyperlipoproteinemia results in a more moderate hypertriglyceridemia due to reduced hepatic uptake of TRL remnants.

Unlike the well known association of apolipoprotein E with plasma cholesterol levels, the relationship between apoE polymorphisms and plasma TG levels is less clear. It is known that the *e*2 allele is somewhat protective, as carriers have lower cholesterol levels and lower incidence of cardiovascular disease, whereas carriers of the *e*4 allele tend to have elevated plasma cholesterol. In contrast, some studies have indicated that effects of apoE polymorphism on plasma TG is often small and not detectible (Davignon *et al*, 1988). In an early meta-analysis (Dallongeville *et al*, 1992) a relationship was found between apoE polymorphisms and plasma TG, although there was more variation than that seen for cholesterol levels. For instance, TG values were generally higher in the *e*2 carriers and in subjects with the *e*4/*e*3 genotype than in those with the *e*3/*e*3 genotype. Interestingly, the strongest relationship was found with the *e*4/*e*3 genotype, and in combination with higher cholesterol and lower HDL-cholesterol levels in this group, a higher cardiovascular disease risk may be indicated than in the other genotypes (Dallongeville *et al*, 1992). Other studies have shown that the *e*2/*e*2 phenotype has a slower plasma clearance of TG-rich remnants and defective lipolysis. Studies examining an association between the *e*4 phenotype and dietary responsiveness have been inconclusive.

Polymorphisms of the apoB *Msp*I and apoB *Bsp* 1261I genes have previously been associated with a greater decrease in plasma TG following a switch from a low fat to high fat diet (Rantala *et al*, 2000).

The fatty acid binding protein (FABP) family is thought to be involved in FA transport. The isoform expressed by enterocytes is the intestinal FABP isoform (I-FABP), encoded by the FABP2 gene. A common polymorphism in exon 2 of the FABP2 gene exists, resulting in an alanine (Ala54) to threonine (Thr54) exchange. This polymorphism seems to be associated with elevated fasting TG levels (Ribalta *et al*, 2005). In response to feeding, the Thr54 allele seems to result in a higher postprandial TG (Agren *et al*, 1998), plasma free fatty acid (Pratley *et al*, 2000) and plasma insulin (Agren *et al*, 1998) response. As previously mentioned, an association of the polymorphism with postprandial TG may depend on a combination with other genotypes. For instance, research has indicated that homozygous Thr54 FABP only affects TG response when associated with a specific FABP2 promoter (Helwig *et al*, 2007). It has also been shown to be triglyceridemic when associated with polymorphisms of microsomal triglyceride transfer protein (Gastaldi *et al*, 2007) and PPAR genes, in combination with APOE2 (Ribalta *et al*, 2005), and that in some cases the effects are sex-specific.

Enzyme Regulation

Changes in the enzymes involved in fat metabolism are due to nutritional regulation of gene transcription, RNA transport from the nucleus and subsequent processing, as well as some protein synthesis and post-translational modification (Sul & Wang, 1998). Examples of alterations in lipogenic enzymes include ACC, FAS, DGAT and GPAT.

ACC is subject to allosteric regulation, activation by citrate, and inhibition by fatty acyl-CoA and post-translational modification (Sul & Wang, 1998;Leahy *et al*, 1999). Conversely, FAS and mitochondrial GPAT are thought to be regulated by control of transcription rates (Sul & Wang, 1998). Diacylglycerol acyltransferase (DGAT) activity seems to be regulated nutritionally and hormonally by gene transcription and protein modification. Protein modification occurs by phosphorylation by AMP-activated kinase (AMPK), a sensor of cellular energy supply. As cellular ATP is depleted, AMPK increases and GPAT activity and TG synthesis are inhibited. Concurrently, AMPK downregulates ACC, which results in decreased production of malonyl CoA, further "pushing the system" away from lipogenesis and towards fatty acid oxidation (Coleman & Lee, 2004).

Transcription Factors

De novo lipogenesis is known to occur primarily in the fed state and is controlled by several transcription factors. Most important in regulating the expression of lipogenic genes is the sterol regulatory element binding protein (SREBP). SREBPs are involved in energy homeostasis by regulating both lipogenesis and cholesterogenesis (Eberle *et al*, 2004). Other nuclear factors include liver X receptor (LXR), retinoid X receptor (RXR) and peroxisome-proliferator-activated receptors (PPARs)(Brownsey *et al*, 2006).

Transgenic mice that overexpress nSREBP-1c show a 6-fold increase in hepatic fatty acid synthesis, whereas knockout mice show a 50% reduction (Horton, 2002). According to a recent review (Blasiole *et al*, 2007), the SREBP-1c isoform upregulates virtually all enzymes in FA synthesis as well as enzymes that supply acetyl-CoA units and reducing equivalents to the pathway. Further, SREBP-1c is induced by insulin, accounting for the lipogenic effect of chronic hyperinsulinemia. SREBP-1 may also be involved in the transcription of long-chain fatty acyl-CoA elongase, mitochondrial GPAT and SCD-1 (Coleman & Lee, 2004). It has been suggested that SREBP-1 is not needed for basal expression of lipogenic genes, but may be required for adaptation to both short and long term diet changes. The concentration of mature hepatic SREBP-1 has been shown to occur between 5 and 24 h of hormone treatment. Since FAS, ACC, ATP-citrate lyase, SCD and spot 14 contain functional steroid response elements; it is possible that the increase in SREBP-1 abundance by T3 is a mechanism of stimulation of these genes (Zhang *et al*, 2003).

In vitro and in vivo studies have shown that insulin stimulates FA synthesis in the liver by inducing transcription of SREBP-1c (Horton, 2002) and LXR. The liver X receptor-a (LXRa) is a nuclear receptor for oxysterols and regulates lipogenesis through the induction of SREBP-1c expression. LXRs influence lipogenesis by two mechanisms, indirectly through SREBP-1c or more directly by binding to gene promoters of lipogenic genes (Matsuzaka *et al*, 2002;Yoshikawa *et al*, 2002). It is thought that when the cellular sterol concentration increases, LXR activates SREBP-1 thereby increasing the synthesis of oleate for cholesterol esters. The suppression of plasma TG by fish oil may be partially through competitive inhibition of LXR activation combined with an increase in the rate of SREBP-1 degradation (Coleman & Lee, 2004).

The more recently discovered carbohydrate response element binding protein (ChREBP) also upregulates lipogenic gene expression. The promoters of many lipogenic genes contain a response element to ChREBP called ChoRE (Ishii *et al*, 2004). ChREBP is activated through the formation xylulose-5-phosphate in the pentose shunt following glucose uptake and is also a target gene of LXR (reviewed in Blasiole *et al*, 2007). High glucose and insulin concentrations stimulate ChREBP gene expression (Dentin *et al*, 2004) and translocation (Kawaguchi *et al*, 2002). In the liver, high glucose activates ChREBP expression independently of insulin (Ishii *et al*, 2004) and this results in the induction of lipogenic genes (Dentin *et al*, 2004;Ishii *et al*, 2004). The activity of ChREBP seems to be reduced in liver extracts from rats fed high-fat diets versus high-carbohydrate fed rats (Kawaguchi *et al*, 2002). It has been hypothesized that the downregulation of ChREBP and SREBP-1c together by low carbohydrate/low insulin/high PUFA may partly explain the shift in hepatic lipid metabolism from lipid synthesis and storage to oxidation associated with the ingestion of polyunsaturated FAs (Dentin *et al*, 2005).

PPARs are members of the superfamily of nuclear hormone receptors that act as transcription factors, signaling the genome from lipid-soluble factors (i.e. hormones, vitamins, and FAs). Fibrates are a class of drug known to be hypotriglyceridemic by acting as PPARα agonists. Administration of fibrates results in decreased TG-rich lipoproteins, LDL cholesterol and increased HDL cholesterol levels. PPARα is activated by fibrates and FAs, particularly in fasted animals. Clinical use of fibrates decreases TG, but the effect on enzymes of TG synthesis is not clear (Coleman & Lee, 2004). However, fibrates seem to have an effect on LPL and apoC-III expression, ultimately resulting in increased lipoprotein lipolysis. In addition to inducing lipolysis, fibrates are thought to increase hepatic FA uptake and reduce hepatic triglyceride production (Staels *et al*, 1998). Fibrate treatment in mice may also indirectly affect lipid metabolism through SREBP-1c. Although administration of the drug does not seem to affect SREBP-1c expression, it is thought to act post-translationally or at the transcriptional level by proteolytic cleavage of the membrane-bound precursor of SREBP (Knight *et al*, 2005).

1.3 HYPERTRIGLYCERIDEMIA

Hypertriglyceridemia has been defined as fasting plasma TG greater than 2.3 mmol/L in adults, or 1.6 mmol/L in those less than 20 years of age (Mancini *et al*, 1991). The most current National Cholesterol Education Program guidelines as put forth by the American Heart Association (Buse *et al*, 2007) for fasting plasma triglycerides are: normal is <1.7 mmol/L, borderline-high TG is 1.7 to 2.2 mmol/L, high is 2.3 to 5.6 mmol/L and 5.6 mmol/L or higher is considered very high. High TG levels are directly associated with relative weight and age (in both sexes), oral contraceptive use (in women), and diabetes mellitus development (Castelli, 1986). Hypertriglyceridemia indicates an excess of serum TG-rich lipoproteins, including VLDL and chylomicrons, as well as the remnants of these lipoproteins (Ooi & Ooi, 1998).

1.3.1 RISK FACTOR FOR CARDIOVASCULAR DISEASE

Fasting Plasma Triglycerides

The significance of elevated plasma TGs to cardiovascular disease has long been controversial. This is due to the close association of high TG to other cardiovascular disease risk factors, which brought in question whether it was an independent risk factor or perhaps just a marker. The Framingham Heart Study showed that individuals with elevated TG are at greater cardiac risk if total cholesterol to HDL ratio is low (<3.5)(Castelli, 1986). However, several studies including the Munster Heart Study, have shown that high TG concentrations increase the risk of major cardiac events independently of HDL or LDL cholesterol concentrations (Assmann *et al*, 1998;Tai *et al*, 1999).

It has been suggested that lowering TGs is important in decreasing the incidence of coronary disease (Sprecher, 1998), however further clinical trials are needed in order to elucidate the reduction in risk (Hokanson & Austin, 1996;Austin *et al*, 1998). Targets for TG are not provided in many guidelines because of the lack of clinical trial data supporting specific recommendations, however optimal TG level is considered to be <1.5 mmol/L. At this level there are fewer associated metabolic abnormalities such as low HDL cholesterol, small dense LDL particles and postprandial lipemia (Leiter *et al*, 2006). This lipid combination is often referred to as "atherogenic dyslipidemia" commonly associated with metabolic syndrome X, which predisposes individuals to both diabetes and cardiovascular disease. Specifically, the "lipid triad" consists of an elevated plasma TG level (>1.7 mmol/L), reduced HDL cholesterol level (<1.0 mmol/L for men; <1.3 mmol/L for women), and a relative excess of small, dense LDL particles that occur with total LDL cholesterol levels that are generally considered normal (Fletcher *et al*, 2005).

Postprandial Lipemia

It is now evident that the postprandial state may be more important than the fasting state in determining the atherogenicity (Krauss, 1998) and cardiovascular disease risk (Patsch *et al*, 1992) associated with TG-rich lipoproteins. Research in postprandial TG metabolism is beginning to expand due to the atherogenic role of remnant lipoproteins but also because the postprandial state is more physiologically relevant than the fasting state in Western society today. Advances in methodologies such as stable isotopes have allowed a more accurate analysis of how the body handles TG in response to a variety of metabolic situations.

Correlations have been drawn between fasting TG levels and plasma TG following a fat load. It was demonstrated that both fasting and postprandial TG were elevated in patients with cardiovascular disease versus those without, however the rise in TG following a fat load was similar, albeit with a wide variability in response. This led researchers to conclude that there was no clinical benefit to measuring postprandial TG (Schaefer *et al*, 2001). Subjects with insulin resistance appear to experience postprandial lipemia of a greater magnitude and duration than insulin sensitive individuals. ApoB-48 and apoB-100 levels tend to be elevated in the diabetic state, indicating increased exposure to atherogenic remnant particles for a longer period of time. It is not known what elevation in particles is clinically relevant. It is possible that a fat load disguises small differences in TG handling, including differences in particle number and clearance rates that may be relevant to cardiovascular disease development over time. Perhaps it would be more relevant to the physiological state to have test meals that resemble what would usually be consumed in order to directly examine postprandial TG metabolism.

1.3.2 PROPOSED CAUSES OF HYPERTRIGLYCERIDEMIA

According to the latest AHA Scientific Statement on Managing Abnormal Blood Lipids (Fletcher *et al*, 2005) there are a number of underlying causes of elevated TGs. These are listed as overweight and obesity, physical inactivity, cigarette smoking, excess alcohol consumption, high-carbohydrate diets (>60% of total energy), genetic predisposition and other diseases such as type 2 diabetes mellitus, chronic renal failure, and nephrotic syndrome.

Knowing the underlying mechanism contributing to each "cause" is important in determining the most effective treatment for any pathology. The root cause of elevated plasma TGs is shared by a dual defect in TG metabolism; hepatic overproduction of VLDL-TG combined with defective lipolysis of VLDL-TG (Mostaza *et al*, 1998). This is consistent with the hypothesis that there is a failure of clearance to "keep up" with production (Huff &

Nestel, 1982). An overproduction of chylomicrons following a meal further aggravates the ability of the body to clear TG as chylomicron-TG competes with VLDL-TG for lipolysis and uptake by the liver.

Fat- vs. Carbohydrate-Induced Lipemia

Fat-induced lipemia is described as a primary hyperlipidemia, which absolves as a result of dietary fat reduction or over time as the plasma clears. Prolonged postprandial lipemia can be induced by a large fat load, and usually involves a combination of long residence time of chylomicron and VLDL remnants in circulation and increased liver synthesis of VLDL (Mero *et al*, 1998). Alternatively, carbohydrate-induced lipemia occurs when subjects are switched to a low fat/high carbohydrate diet (Parks & Hellerstein, 2000).

In a review of carbohydrate-induced lipemia, Parks and Hellerstein (2000) state that the health effects of this phenomenon are "among the most controversial and important issues in public health nutrition today." Briefly, they note that carbohydrate-induced lipemia is common when obesity is prevalent and diets are hypercaloric, and is pronounced when lipemia is maintained after subjects are switched to low fat/high carbohydrate diets. The basic trend indicates that the more carbohydrate increases and fat decreases in the diet, the more TG levels increase. It seems that carbohydrate induction could be the result of increased production of VLDL-TG from the conversion of carbohydrate to fat through the *de novo* pathway in the liver. However, this is complicated by indications that responses to low fat/high carbohydrate diets are heterogeneous. Studies using labeled precursors of FA synthesis have indicated that low fat diets result in decreased TG clearance and/or increased synthesis with large individual variations in response (Hudgins, 2000). The extent of carbohydrate induction depends on several factors such as baseline lipid concentration, age, body mass index, as well as the type of carbohydrate fed (mono- or polysaccharide, fiber). Sucrose and fructose tend to increase TG levels the most drastically, while fibre tends to have a beneficial effect (Parks & Hellerstein, 2000).

The length of time that TG levels remain elevated with high carbohydrate diets is unknown. Some evidence suggests that the effect is only transitory and can be prevented if dietary fat is reduced gradually and replaced with whole food carbohydrate rather than sugars (Parks

& Hellerstein, 2000). Indeed, it may be important that the carbohydrate is low in glycemic index (Wolever *et al*, 1991). It has also been hypothesized that dietary fat level can prevent carbohydrate-induced hypertriglyceridemia. The differences in the hypertriglyceridemic effect of the type of carbohydrate (fructose vs. glucose vs. starch) fed seems to be less pronounced with higher fat diets up to 30% of energy (Herzberg, 1991). A two-year study involving hyperlipidemic subjects indicated that carbohydrate induction lasts as long as the diet and may have a threshold for some subjects at normal carbohydrate intakes (Knopp *et al*, 2000). It has also been found that in diabetic subjects the effect seems more persistent, lasting at least several months (Garg *et al*, 1994).

High carbohydrate diets have been tested in obese versus lean individuals. Researchers (Hudgins *et al*, 2000) measured FA synthesis and found that despite a two-fold elevation in insulin and reduced glucagon levels in the obese subjects, there were no significant differences in dietary effects. Although responses were variable, the high carbohydrate diet increased de novo synthesis of fatty acids and this correlated with the increase in TG levels (Hudgins *et al*, 2000).

A 6 month study in hyperlipidemic free-living subjects showed that both a high carbohydrate/high fibre and a high unsaturated fat diet significantly reduced LDL cholesterol levels and fasting and postprandial concentrations of TG, without negatively affecting HDL cholesterol, plasma glucose or insulin concentrations. The researchers attributed this to the high fibre content in the low fat diet, and the high monounsaturated fat level in the higher fat diet, as well as to the length of the study (Rivallese *et al*, 1994).

The effect of low fat/high carbohydrate diets on postprandial lipemia is unclear. Much of the research on postprandial TG metabolism has utilized a non-standardized and often very large fat load to study TG-rich lipoprotein production and clearance. It was thought that overwhelming the system was an effective way to determine how well it was able to handle a fat, in a similar fashion to an oral glucose tolerance test. As a result, not many postprandial studies have shown the lipid response to a lower fat higher carbohydrate and/or mixed meal. Of the research available, it has been indicated that low fat/high carbohydrate intake elicits lower postprandial TG, but results in higher fasting levels when fed chronically. However, other research seems to show that high carbohydrate also results in higher postprandial TG (Koutsari *et al*, 2000) and the accumulation of TRLs (Abbasi *et al*, 2000), particularly in insulin resistant individuals (Chen *et al*, 1995).

It has been suggested that the metabolic interaction between carbohydrate and fat forms the basis of carbohydrate-induced hypertriglyceridemia (Chong *et al*, 2007). The addition of 50g fructose to a 5g fat load was shown to increase the plasma TG-rich lipoprotein fraction to a level three times greater than a fat load of 80g (Jeppesen *et al*, 1995). It is possible that the ingestion of fat in combination with carbohydrate affects the handling of free FA and substrate oxidation differently from carbohydrate or fat ingested alone (Griffiths *et al*, 1994). Since macronutrients are not consumed individually, it is important to determine the dietary proportion and composition most beneficial in minimizing plasma TG concentration.

De novo Lipogenesis

Animal studies indicate that increased de novo lipogenesis may play an important role in the pathogenesis of hypertriglyceridemia (Šebökováat *et al*, 1996) and onset of obesity (Cheema & Clandinin, 1995). However, some researchers have claimed that "the fatty acid biosynthetic pathway is quantitatively minor in humans except when a large excess of carbohydrate energy is consumed" (Hellerstein, 1999). As will be further discussed, this is a value judgment that requires a physiological perspective. It is known that de novo lipogenesis has the potential to make a large contribution to VLDL-TG in animals, whereas the human situation is much less clear. Quantitative claims aside, isotope studies reveal a relationship between plasma TG level and hepatic de novo lipogenesis (Konrad *et al*, 1998). The capacity for lipogenesis in humans varies considerably and in some individuals fatty acid synthesis may occur at high enough rates to result in increased plasma and adipose tissue levels of TG. This may be particularly accentuated in those that consume lipogenic diets or those with physiological defects (Hillgartner *et al*, 1995).

It is becoming evident that availability of fat may be a major driver of VLDL overproduction (Adiels *et al*, 2006). Therefore, lowering the rate of lipogenesis may aid in reducing plasma TG levels. Also, the composition of endogenously synthesized TG tends to be more saturated, possibly leading to depleted plasma and tissue essential fatty acids (Hudgins *et al*, 1996). One could conjecture that if de novo lipogenesis changes the lipid composition of the hepatocyte, this might exert a unique influence on VLDL synthesis and secretion.

Insulin Resistance and Diabetes

Fasting hypertriglyceridemia may be the most characteristic metabolic abnormality in insulin-resistant/hyperinsulinemic individuals. As previously stated, postprandial lipemia also tends to be more accentuated and persistent in insulin resistance and diabetes.

Although the availability of fat may be a major driver of VLDL overproduction (Adiels *et al*, 2006), it is thought that insulin controls VLDL secretion (Lewis *et al*, 1995;Malmstrom *et al*, 1997b). The state of insulin resistance at the level of the liver may be associated with increased lipogenesis (Parks, 2001). Hyperinsulinemia normally acts to suppress VLDL1 production but in diabetes this suppressive effect seems to be absent, even when free FA release is blunted (Malmstrom *et al*, 1997a). Originally it was thought that the primary effect of insulin resistance was a resistance of the anti-lipolytic effect of insulin on adipose tissue. This reduction of plasma free FA levels reduced the availability of free FA to the liver as a driver of VLDL synthesis. However, increased free FA availability is not fully responsible for producing the high VLDL production rates in insulin resistance and type 2 diabetes. When insulin resistance results in chronically elevated glucose and insulin levels, VLDL production is also elevated due to stimulation by glucose and resistance of the liver to the inhibitory effect of insulin on VLDL production.

It has been speculated that VLDL oversecretion may depend on a number of factors functioning together in some capacity, all associated with insulin resistance. These include the presence of peripheral tissue insulin resistance, hepatic insulin resistance or overinsulinization, and/or visceral obesity (Lewis *et al*, 2002). Hypertriglyceridemia, insulin resistance and visceral obesity tend to occur together along with other metabolic abnormalities, referred to as metabolic syndrome X or insulin resistance syndrome.

As previously mentioned, high carbohydrate diets seem to be particularly detrimental to hypertriglyceridemia in insulin resistant states (Reaven, 2005). Low fat high carbohydrate diets tend to increase hepatic VLDL-TG synthesis and secretion, resulting in higher fasting TG concentrations in patients with type 2 diabetes (Garg *et al*, 1994). High carbohydrate intake potentially leads to hyperglycemia, which in combination with hyperinsulinemia, increases lipogenesis. Concurrently, insulin is unable to suppress VLDL secretion or free FA release into plasma. As a result, low fat/high carbohydrate diets in insulin resistant and hyperinsulinemic individuals will both increase fasting plasma TG concentration and accentuate the daylong accumulation of TG-rich remnant lipoproteins (Reaven, 2005).

Type 2 diabetes patients tend to have overall higher total and VLDL cholesterol levels, and the VLDLs seem to be of altered composition (Howard, 1987). Insulin resistance has been pinpointed as the key determinant of these modifications, as a result of impaired insulin action on VLDL (Bioletto *et al*, 2000). Insulin resistant subjects also seem to be more prone to carbohydrate-induced hypertriglyceridemia (Parks & Hellerstein, 2000), however it is not known whether the mechanisms underlying carbohydrate-induced hypertriglyceridemia differ in patients with type 2 diabetes. Research has indicated that high carbohydrate intake does not affect postheparin LPL or hepatic lipase activities, therefore increased hepatic secretion of VLDL-TG may play a greater role than reduced lipolysis/diminished clearance of TG-rich lipoproteins (Blades & Garg, 1995). Previously, it was shown that apoB and TG production rates tend to be higher than that of non-diabetics but that clearance of VLDL apoB and TG is also decreased in diabetes (Howard, 1987). VLDL apoB and TG production have been positively correlated with plasma insulin levels and catabolism negatively correlated with plasma glucose levels (Howard, 1987). The development of hypertriglyceridemia as a result of altered VLDL metabolism may factor into altered LDL profiles as well (Bioletto *et al*, 2000).

According to a recent review, SREBPs have been "incriminated in the development of human metabolic physiopathology such as obesity, type 2 diabetes, dyslipidemia, atherosclerosis, global syndrome X and lipodystrophy" (Eberle *et al*, 2004). Several studies have demonstrated that the adipose tissue of obese and type 2 diabetic patients express lower levels of SREBP-1c mRNA compared to lean subjects and that expression increases with weight loss and insulin sensitivity (Ducluzeau *et al*, 2001;Diraison *et al*, 2002). In insulin resistant mice despite IRS-2 deficiency, it seems that insulin continues to stimulate SREBP-1c transcription and expression in the liver resulting in lipogenic gene expression, FA synthesis and TG accumulation. The combination of insulin resistance (inappropriate gluconeogenesis) and insulin sensitivity (elevated lipogenesis) launches a vicious cycle that aggravates hyperinsulinemia and insulin resistance in lipodystrophic and ob/ob mice (Shimomura *et al*, 2000).

1.3.3 TREATMENT OF HYPERTRIGLYCERIDEMIA

It is important to know the origin of hypertriglyceridemia in order to define a suitable intervention. For instance, it has been suggested that increased VLDL secretion can be treated with pharmaceuticals, whereas reduced clearance of VLDL can be treated by "nontherapeutic regimens such as exercise training" (Parks & Hellerstein, 2000).

Diet

Adiposity is argued to be the main nutrition-related influence associated with atherogenic dyslipidemia and Adult Treatment Panel III recommends that treatment be focused on reducing TG levels. Consequently, for these individuals, weight loss is a primary goal as a means to lower TG levels (Fletcher *et al*, 2005) and this led to low fat/high carbohydrate diet recommendations. The question of cause or effect could be raised here.

There is also general agreement (Fletcher *et al*, 2005) that the major dietary determinant of elevated TGs in atherogenic dyslipidemia is carbohydrate, particularly high glycemic carbohydrates. Fat intake is recommended at 25- 35% of calories along with complex carbohydrates and high fibre intake to facilitate TG lowering, as well as increasing HDL cholesterol and converting LDL into larger, more buoyant particles (Riccardi & Parillo, 1993). Consistent evidence has also shown the effectiveness of high monounsaturated fat diets (Garg, 1998), as well as those rich in mixed unsaturated fats (Pieke *et al*, 2000), in lowering serum TG and LDL levels, while increasing or not adversely affecting HDL levels.

Omega-3 fatty acids are also well-recognized in the management of hypertriglyceridemia (Fletcher *et al*, 2005). It has been suggested that increasing long chain n-3 FAs from fish or fish oil, while reducing saturated fat and cholesterol may show the most benefit (Connor *et al*, 1993). In normolipidemic subjects, fish oil intake prevents and reverses carbohydrate-induced hypertriglyceridemia. Elevated TG levels in genetically hypertriglyceridemic rats were decreased with fish oil supplementation, in part by suppressing gene expression of FAS and malic enzyme in the liver (Sebokova *et al*, 1996). Both eicosapentaenoic acid and docosahexaenoic acid have been shown to decrease TG and increase fasting insulin in healthy and diabetic individuals, even though there are some differing effects on other serum lipids and fasting glucose concentrations (Mori *et al*, 2000). The hypotriglyceridemic effect of fish oil seems to be more pronounced in hypertriglyceridemic individuals due to increased LPL activity in muscle, increased removal and decreased synthesis of TG by the liver (Herzberg, 1991) and reduced rate of hepatic production of VLDL-TG apoB (Chan *et al*, 2003). The ratio of long chain n-3 FAs to other dietary FAs is important. It has been observed that the prevalence of type 2 diabetes increases as the ratio of n-6 FAs to n-3 FAs increases in populations (Raheja *et al*, 1993).

Current Guidelines and Recommendations

Unfortunately, interventions for reducing elevated plasma TG concentration have not been as clearly defined as those for other lipids. Unlike LDL-cholesterol targets, which have been thoroughly researched by pharmaceutical companies in developing statin treatments, specific targets for TG are not provided due to a lack of clinical data. It has been stated by the American Heart Association Nutrition Committee that because a moderate inverse relationship exists between TG and HDL cholesterol concentrations, the determinants should be the same for high TG as for low HDL cholesterol (i.e. hyperglycemia, diabetes, hypertriglyceridemia, very low-fat diets, excess body weight). A level of 1.5 mmol/L is specified due to its use in the classification of metabolic syndrome (Lichtenstein *et al*, 2006). This level of TG has also been suggested to be optimal by the Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, since below this level of hypertriglyceridemia there are fewer associated metabolic abnormalities (i.e. low HDL-C, small dense LDL particles and postprandial lipemia)(Leiter *et al*, 2006).

There seems to be agreement that for the general population, after achieving the LDL cholesterol goal, TG levels of 1.7 to 2.2 mmol/L should be treated with therapeutic lifestyle changes (Pearson *et al*, 2002). The AHA suggests losing weight (if indicated), replacing saturated fat with monounsaturated and polyunsaturated fats, substituting fish high in omega-3 fatty acids for meats high in saturated fat, reduction of trans fat, cholesterol and alcohol intake, as well as increasing physical activity. Recently, the AHA and the American Diabetes Association (ADA) put forth a scientific statement addressing the primary prevention of cardiovascular diseases in people with diabetes. It was recognized that for people with diabetes, TG-rich lipoproteins and VLDLs are elevated and should be a secondary target of lipid-lowering therapy (after LDL cholesterol) as serum TGs are a "surrogate for atherogenic triglyceride-rich lipoproteins" (Buse *et al*, 2007). The two agencies suggest different approaches to the management of HDL- and TG-associated cardiovascular disease risk. The AHA recommends that in patients with TG levels of 2.2 to 5.6 mmol/L, a non–HDL cholesterol goal of 3.4 mmol/L is a secondary target. If TGs are >5.6 mmol/L, the therapeutic options recommended include fibrates or niacin and treatment of LDL cholesterol after TG-lowering therapy. The ADA suggests lowering TGs to 1.7 mmol/L and raising HDL cholesterol to 1.15 mmol/L in men, and 1.3 mmol/L in women (Buse *et al*, 2007).

1.4. INSULIN RESISTANCE AND DIABETES

1.4.1 INSULIN RESISTANCE AND PREDIABETES

The conventional classification of insulin resistance is "the impaired ability of skeletal muscle cells and other types of cells to remove glucose from the blood under the action of insulin" (Lipkin, 1999). The impairment of insulin action extends not only to stimulation of glucose uptake, but also inhibition of glucose output and lipolysis (Storlien *et al*, 2000). Insulin resistance is commonly present in many metabolic disorders, including type 2 diabetes, hypertension, dyslipidemia, and cardiovascular disease. Many insulin resistant individuals show no apparent symptoms, but retain impaired fasting blood glucose levels and hyperinsulinemia (Lipkin, 1999).

The term 'prediabetes' is used to describe impaired blood glucose levels because it places individuals at risk of developing diabetes and its complications. The Canadian Diabetes Association (CDA) defines impaired fasting glucose (IFG) as a fasting plasma glucose of >6.1 mmol/L and impaired glucose tolerance (IGT) as a glucose level of 7.8–11.0 mmol/L following a 2 hour 75g oral glucose tolerance test (Canadian Diabetes Association Clinical Practice Guideline Expert Committee, 2003). The American Diabetes Association (ADA) has since modified the 2001 guidelines to define IFG as a fasting plasma glucose of >100 mg/dL (5.6 mmol/L) (2006). The ADA states that elevated blood glucose levels below the threshold for diabetes have clinical consequences, however not all individuals with prediabetes will progress to diabetes. Identifying people with prediabetes may indicate those who would benefit from cardiovascular disease risk factor modification, particularly in the context of the metabolic syndrome. The CDA recognizes that lifestyle interventions are highly effective in delaying or preventing the onset of diabetes in people with IGT, however reductions in cardiovascular disease and total mortality have not been demonstrated (Canadian Diabetes Association Clinical Practice Guideline Expert Committee, 2003).

Development of Insulin Resistance

Factors that tend to be associated with insulin resistance are obesity, aging, sedentary lifestyle, high-fat diets, stress, altered androgen metabolism, increased cytokines as well as skeletal muscle fiber, membrane and capillary changes (Kelley, 2000). Insulin resistance has a strong hereditary component, however the genetic mutations involved are described as elusive, numerous and difficult to differentiate (Kelley, 2000). The fact that insulin resistance influences several metabolic processes complicates the issue. There is speculation as to whether it is actually the cause or the result of many of the metabolic abnormalities associated with it.

Insulin resistance can occur at several sites, and the metabolic effect depends on the location of the insulin resistant tissue. Muscle and liver insulin resistance contributes to the hyperglycemia in type 2 diabetes. Although muscle accounts for 70-80% of insulin-stimulated glucose disposal, the liver may have a greater role in influencing glucose concentrations due to hepatic glucose output (Whitelaw & Gilbey, 1998). Recently the role of adipose tissue in insulin resistance has received more attention. Adipose tissue insulin resistance relates to the observation that plasma free FA levels are increased in type 2 diabetes, most likely due to the inability of insulin to suppress lipolysis in this tissue (Bergman & Mittelman, 1998).

High fat diets have been implicated in the etiology of insulin resistance for many years, mostly due to the rapid induction of obesity and insulin resistance in many animal models when switched to a high fat/high sucrose diet. Research in rats has indicated that high fat diets induce muscle insulin resistance or a reduction in insulin stimulated glucose uptake (Han *et al*, 1997;Wilkes *et al*, 1998). The underlying mechanism for this effect has not been fully elucidated. Elevated plasma free FAs are thought to induce insulin resistance by reducing muscle glycogen synthesis and glucose oxidation due to inhibition of glucose transport/phosphorylation (Roden *et al*, 1996). Another mechanism suggested is an impairment of one or more of the steps involved in the GLUT4 translocation process (Hansen *et al*, 1998).

The type of FA available is important because saturated FAs appear to cause insulin resistance whereas unsaturated fatty acids seem to be protective or even improve insulin sensitivity (Clandinin *et al*, 1993). High saturated fat intake is believed to result in more saturated membrane lipids (Vessby, 1995). More recently, it was shown in vitro that saturated fat induced skeletal muscle insulin resistance by inducing intramyocellular accumulation of fatty acyl-CoA metabolites (Adams *et al*, 2004). The mechanism is not completely understood, however saturated fatty acids are less readily oxidized and accumulate as diacyglycerol and ceramide, whereas mono- and polyunsaturated fats accumulate as intramyocellular TG or free FAs (Lee *et al*, 2006).

Because of the close association between obesity, insulin resistance and elevated plasma free FAs, it was thought that free FAs were important in the development of insulin resistance. An increase in plasma free FA level results in oxidative stress, inflammation and vascular changes. The metabolic syndrome, which is closely tied to insulin resistance, has been recognized as an inflammatory disease manifesting in elevated inflammatory markers such as CRP, PAI-1, IL-6 and TNF-α (Garg *et al*, 2003). It has since been postulated that the pro-inflammatory state induces insulin resistance, leading to clinical and biochemical manifestations of the metabolic syndrome. This is because macronutrient intake is supposedly pro-inflammatory and insulin anti-inflammatory; therefore resistance to insulin action would promote further inflammation. The resultant effects exacerbate the situation through an increase in free FA concentration and a vicious circle of metabolic responses (Dandona *et al*, 2005).

1.4.2 TYPE 2 DIABETES

The number of people diagnosed with diabetes mellitus worldwide has experienced an "explosive increase" attributed mostly to changes in the human environment, behaviour and lifestyle, as well as rising obesity rates and aging populations. In 2001, the global estimate of diabetes incidence was 150 million and was projected to rise to 220 million in 2010 and 300 million by 2025. Of this, type 2 diabetes accounts for over 90% of cases (Zimmet *et al*, 2001). In 1998, 5% of Canadians had been diagnosed with diabetes, and this number was expected to double by the year 2010 to an estimated 3 million people (Meltzer *et al*, 1998). These are likely underestimates because recent research has indicated that the prevalence of diabetes in Ontario has increased steadily from 1995 to 2005, by an average of 6.2% per year. This represents a 69% increase since 1995, already exceeding the 60% global increase estimate and 65% Canadian increase projected for 1995 to 2030 (Lipscombe & Hux, 2007).

Etiology of Diabetes

The 2003 Clinical Practice Guidelines from the Canadian Diabetes Association defined diabetes as having a FPG of ≥7.0 mmol/L (no caloric intake for at least 8 hours) or a casual plasma glucose of ≥11.1 mmol/L plus the symptoms of diabetes (any time of the day irregardless of last meal). The classic symptoms of diabetes are polyuria, polydipsia and unexplained weight loss or plasma glucose 2 hour after a 75g oral glucose tolerance test of ≥11.1 mmol/L (Canadian Diabetes Association Clinical Practice Guideline Expert Committee, 2003). Type 1 diabetes develops in childhood as insulin deficiency due to autoimmune-mediated destruction of pancreatic-cell islets (Zimmet *et al*, 2001). Type 2 diabetes typically develops in adulthood as a result of abnormalities in insulin secretion, insulin action, or both. It is only those individuals unable to sustain a state of 'compensatory hypersinsulinemia' due to impaired insulin secretory capacity that actually develop type 2 diabetes (Reaven, 2005). The risk for developing type 2 diabetes is higher in individuals with established impaired fasting glucose or impaired glucose tolerance (Shaten *et al*, 1993). The inability of the β-cells to secrete sufficient compensatory insulin does not adequately account for the pathogenesis of hyperglycemia.

Type 2 diabetes patients have chronically high plasma free FA levels and this may indicate that adipose tissue is also insulin resistant (Bergman & Mittelman, 1998), rendering elevated insulin levels unable to suppress free FA release from adipose tissue (Mostaza *et al*, 1998). These elevated plasma free FAs have multiple metabolic effects, such as a decrease in insulin-stimulated glucose uptake, a stimulation of hepatic free FA oxidation and glucose production, and an inhibition of β-cell response to glucose, further aggravating the insulin resistant state (Bergman & Mittelman, 1998).

The presence of hepatic insulin resistance in type 2 diabetes seems to occur at the level of glycogenolysis regulation. Investigations of the acute effects of insulin have indicated that type 2 diabetes patients are resistant to the direct hepatic suppressive effect of insulin, however the indirect peripherally mediated suppression of glucose production may remain intact (Lewis *et al*, 1999). Since insulin normally inhibits TG secretion, hepatic insulin resistance would thereby cause an increase in TG secretion rates (Parks & Hellerstein, 2000).

Macrovascular Complications

The prevalence of hypertriglyceridemia in individuals with type 2 diabetes is >2-3x that of non-diabetics (Temelkova-Kurktschiev *et al*, 1997). In the United States it was reported in 2000 that the median TG level in type 2 diabetes patients was <2.3 mmol/L with 85-95% of patients having TG levels below 4.5 mmol/L (2000). Hypertriglyceridemia is considered an independent risk factor for cardiovascular disease in the general population (Sprecher, 1998) and as an independent risk factor for atherosclerosis in diabetes (Temelkova-Kurktschiev *et al*, 1997). This is due to the atherogenicity of TG-rich lipoproteins, other metabolic changes such as increased metabolic clearance of HDL and smaller denser LDL. Type 2 diabetes patients have 3x the heart disease death rate than non-diabetics with similar cholesterol levels; therefore other factors associated with diabetes must be involved. These include hyperglycemia, dyslipidemia, hypertension, obesity, insulin resistance, hyperinsulinemia, and hemostatic disturbances (Temelkova-Kurktschiev *et al*, 1997). Together, these factors are part of what is commonly referred to as the metabolic syndrome X, first described by Gerald Reaven in 1988 (Reaven, 1988).

Treatment

The pharmacological treatments for type 2 diabetes, as reviewed by Lipkin (1999), focus on normalizing glycemia, with the ultimate goal of minimizing the unwanted side effects and complications associated with diabetes. Some of pharmacological strategies for controlling blood glucose include; promoting insulin release from the pancreas (sulfonylureas), reducing hepatic glucose synthesis (metformin), blocking enzymes of complex carbohydrate digestion (acarbose), sensitizing tissues to insulin action (rosiglitazone) or inducing weight loss (antiobesity drugs). Another treatment, exogenous administration of insulin therapy, acts to suppress hepatic gluconeogenesis. These treatments have some unfortunate effects, such as those induced by insulin administration and sulphonylureas, includinh hyperinsulinemia, hypoglycemia and weight gain, along with their accompanying sequelae (i.e. cardiovascular effects). Lipkin notes that these therapies are additive, should be tailored to individual requirements by a health professional, and are independent of the effects that can be achieved with diet and exercise (Lipkin, 1999).

It is well accepted that the goal of treating dyslipidemias in insulin resistance is prevention of cardiovascular disease. Considerable improvements in HDL cholesterol and TG levels, as well as improved insulin action can be accomplished by weight loss and exercise (Howard, 1999). Some diabetic drugs may also be useful in helping to decrease TG and increase HDL cholesterol levels. It was also indicated that some statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) can reduce TG and LDL cholesterol levels while increasing HDL and reducing coronary events.

1.4.3 MACRONUTRIENTS AND DIETARY RECOMMENDATIONS

A fervently debated area pertaining to diet and insulin resistance is optimal macronutrient balance, particularly with respect to carbohydrate and fat. It is based on the argument that both have the ability to aggravate the diabetic state. As previously outlined, research has shown that high carbohydrate diets can induce hypertriglyceridemia and potentially increase glycemic load in diabetes (Garg *et al*, 1994). Conversely, higher fat diets have been discouraged due to possible adverse effects on body weight (Vessby, 1995) and cholesterol levels (Norum, 1992). Obesity and high LDL-cholesterol levels have been shown to contribute to the diabetic state and subsequent cardiovascular disease risk (Meltzer *et al*, 1998). High fat diets can be detrimental to hypertriglyceridemia in the insulin resistant state, with hyperinsulinemia and unrestrained fatty acid flux potentially contributing to VLDL overproduction (Parks & Hellerstein, 2000).

The dietary recommendations for people with diabetes remain somewhat controversial. The Canadian Diabetes Association states that nutrition management is a key component of the overall health of diabetic individuals. It is emphasized that the nutritional recommendations are basically the same as the general population (*Canada's Food Guide to Healthy Eating*, Health Canada), but that people with diabetes should work with a health care team to meet these guidelines (Wolever *et al*, 1999). Specifically, the Canadian Diabetes Association states that 50-60% of daily energy requirements should come from carbohydrate, total fat should be limited to $\leq$30% of energy (saturated and polyunsaturated fats providing $\leq$10%) and intakes of protein recommended at about 0.86 g/kg/day, fibre at 25-35g/day and consumption of fish with n-3 FAs at least once a week. Recently, intake of monounsaturated fat was suggested where possible. It has also been stated that for diabetic individuals with dyslipidemia, general nutrition management should be the same as for dyslipidemic individuals without diabetes (Wolever *et al*, 1999). Conversely, some experts strongly advise that the dietary recommendations given to diabetic patients be considerably different from that of non-diabetics. The metabolic syndrome that surrounds diabetes would suggest that lipid and lipoprotein metabolism is significantly altered in diabetic states and should be treated as such (Garg *et al*, 1994). It is becoming increasingly evident that the traditional "low fat" diet recommended to reduce cardiovascular disease risk, specifically to replace saturated fat with carbohydrate, may not be beneficial and may be detrimental in some insulin resistant individuals (Reaven, 2005). Low fat diets have not been proven to be more effective in reducing weight or improving lipids than higher fat diets, and in fact may worsen many of the symptoms of the metabolic syndrome.

At the peak of the controversy, an important meta-analysis (Garg, 1998) was performed in light of the low fat dietary recommendations for diabetes patients to determine the effects of diet composition on plasma lipids and lipoproteins. Only randomized, crossover trials, comparing the effects of isoenergetic, weight-maintaining high-monounsaturated-fat diets with high-carbohydrate diets in type 2 diabetes patients were used in the meta-analysis (total=9). It was concluded that the high-monounsaturated fat diets (overall >37% energy from fat and <40% energy from carbohydrate) improved lipoprotein profiles by reducing fasting plasma TG and VLDL concentrations (19% and 22% respectively), while increasing HDL cholesterol slightly with no adverse effects on LDL. This diet also improves the glycemic profile in diabetes patients, which may be due to a reduction of the carbohydrate load in the insulin resistant state. It was concluded that eucaloric high-monounsaturated diets should not induce weight gain and that this dietary approach can be recommended to type 2 diabetes patients.

Many studies since have shown that higher fat diets can be beneficial toward the lipid profiles and glycemic control of type 2 diabetes subjects. The consensus among them is that monounsaturated fat diets have beneficial short and long term effects when compared to eucaloric low fat high carbohydrate diets (Garg *et al*, 1994;Garg, 1998), particularly when the polyunsaturated to saturated fat ratio is also high (Reaven, 2005). The optimal macronutrient content of the diabetic diet has always been debated, however. High carbohydrate diets tend to be favored by some claiming that effects on TG levels are heterogeneous (Mayer-Davis *et al*, 1999), and that many diabetes patients are obese and therefore fat intake should be reduced (Vessby, 1995).

1.5 MEASUREMENT OF LIPOGENESIS

Lipogenesis can be measured *in vivo* by several methods. Animal studies employ more intrusive and hazardous methodology than human studies. This includes radioactive isotope labeling and direct measurements of adipose or liver tissue mass and composition. In humans, the metabolism of fat can be followed using stable isotopes. The tissue used for extraction of the lipid of interest ultimately determines what is being measured. For instance, hepatic de novo fatty acid synthesis can be calculated from VLDL-TG, whereas adipose tissue de novo fatty acid synthesis would require extraction of fat from adipose tissue or perhaps the plasma free FAs released from adipose tissue.

1.5.1 INDIRECT CALORIMETRY

This method can be utilized to calculate substrate oxidation rates using measurements of respiratory gas exchange and urinary nitrogen excretion (Tappy & Schneiter, 1997). After a carbohydrate loading meal, the excess carbohydrate is either utilized for oxidation or stored as glycogen. After the glycogen stores are saturated, carbohydrate is disposed of by increased oxidation rates and de novo lipogenesis (Acheson *et al*, 1988). Indirect calorimetry does not measure flux through the *de novo* pathway and cannot be used to study dietary effects on FA synthesis when energy intake and expenditure are in balance (Hudgins *et al*, 1996). It can only be used to measure net conversion of carbohydrate to fat, since the resulting respiratory quotient indicates that synthesis is greater than oxidation during the sample period in the whole system (Hellerstein, 1999). However, it can be useful in studying metabolic processes when combined with other *in vivo* techniques such as isotopic methods (Simonson & Defronzo, 1990).

1.5.2 STABLE ISOTOPES

These methods employ the use of stable isotopes to measure the flux through a metabolic pathway *in vivo* (Hellerstein, 1999) and therefore rate of *de novo* synthesis rather than net synthesis. This involves labeling a precursor in order to quantify labeled end products of the process being studied. In animal studies, the precursor of cytosolic acetyl CoA can be labeled and if the enrichment is known, the fraction of newly synthesized FA molecules in plasma TG FAs can be calculated. However, the acetyl CoA pool cannot be accessed to determine precursor enrichment in human studies. Methods have been developed to get around this issue in human studies, resulting in two reliable techniques used to directly measure de novo lipogenesis *in vivo.* Mass isotopomer distribution analysis (MIDA) and deuterium incorporation (Hellerstein & Neese, 1999) have been successfully used since the early 1990's to trace de novo lipogenesis in humans.

Mass Isotopomer Distribution Analysis

MIDA can be applied to measure fractional FA synthesis and rate of appearance of plasma free FAs (Brunengraber *et al*, 1997). MIDA is a technique based on combinatorial probabilities and the labeling patterns in intact polymers. Fatty acids are made up of identical repeating monomeric subunits, and therefore considered biological homonuclear polymers (Hellerstein & Neese, 1999).

Even though the number of subunits in a polymer is known and can be measured, the population of assembled polymers will not be of uniform isotopic composition. Difficulty with the methodology arises because the polymers exist as different species with varying amounts of label in subunits, i.e. some species will include no labeled subunits and some will have one or more labeled subunits (Hellerstein & Neese, 1999). Therefore MIDA must rely on combinatorial probabilities. That is, measurement and analysis of mass isotopomer abundance distributions in intact polymers according to a combinatorial probability model, after introduction of a stable isotopically labeled monomeric subunit. For instance, a natural abundance of label of a subunit combine into polymer subunits in a characteristic distribution of molecular species. These proportions can be represented as a frequency and the mass isotopomer pattern measured by mass spectrometry. After correction for natural abundance, degree of enrichment of the precursor pool can be calculated by comparing measured patterns of mass isotopomer abundances with those predicted from theoretical precursor pool enrichments (Hellerstein & Neese, 1999).

In lipogenesis, palmitate mass isotopomer distribution as synthesized from ^{13}C-labeled acetate as a tracer can be used to calculate the enrichment of acetyl-CoA. The true precursor-pool enrichment then allows the precursor-product relationship to be applied and the fraction of newly-synthesized molecules present can be calculated (Parks & Hellerstein, 2006). The excess enrichment over baseline is required in order to determine the precursor enrichment and then the fraction of total lipid resulting from de novo lipogenesis can be calculated from the per-carbon enrichment (Hellerstein *et al*, 1991;Hellerstein & Neese, 1992).

This method assumes that there is a constant enrichment of lipogenic actyl-CoA in all hepatocytes. However these enrichments may decrease across the liver, and therefore fractional hepatic lipogenesis may actually be underestimated using MIDA (Puchowicz *et al*, 1999). Despite this, it is claimed that the single most difficult problem facing the use of MIDA relates to the quantitative accuracy of measurements, that is, the analytic performance of mass spectrometers (Hellerstein & Neese, 1999).

The comparisons between rates of whole-body de novo lipogenesis measured by indirect calorimetry and rates of hepatic lipogenesis of VLDL-TG calculated by MIDA (Aarsland *et al*, 1997) lead to the conclusion that the liver plays a quantitatively minor role in the conversion of surplus carbohydrate to fat and that adipose tissue was probably the main site of fat synthesis (Brunengraber *et al*, 1997).

MIDA has also been applied to determine adipose tissue TG metabolism, but not by use of the ^{13}C label. The expense and inefficient equilibration with the precursor pool make it unsuitable (Murphy, 2006), especially over long periods of time as is required in adipose TG turnover studies. In this case, MIDA and ^2H_2O is used to trace new -glycerol phosphate-derived TG synthesis and turnover in mice, rats (Turner *et al*, 2003) and humans (Strawford *et al*, 2004). It is based on incorporation of hydrogen atoms from ^2H_2O into covalent C-H bonds in the glycerol moiety of acylglycerides thereby reflecting newly assembled TG molecules synthesized during label exposure. This research has shown that over the long term, de novo lipogenesis contributes about 20% to adipose tissue palmitate stores in humans, with substantial interindividual variability (Strawford *et al*, 2004).

Deuterium Incorporation

Techniques involving deuterium labeled water involve ingesting ^2H_2O followed by integration of ^2H into body water and its subsequent incorporation into fatty acids (Brunengraber *et al*, 1997). The incorporation of deuterium into the VLDL-TG above natural abundance can be measured using isotope ratio mass spectrometry and from this the rate of TG synthesis by the liver can be determined (Leitch & Jones, 1991).

During *de novo* lipogenesis, deuterium is incorporated into FAs directly from body water or during synthesis and elongation. The palmitate molecule has 31 hydrogens that can be labeled, the hydrogens from odd-numbered carbons come from NADPH and one from each of the even-numbered carbons is from water. The methyl hydrogens and other hydrogens on the even carbons come from acetyl-coA (Murphy, 2006). The enrichment of acetyl-coA and NADPH will depend on their source, which may vary from tissue to tissue or depend on metabolic state. For instance, NADPH derived from the malic enzyme pathway will be in exchange with the body water but not NADPH from the pentose cycle (Murphy, 2006). On average, it has been shown that three deuterium atoms per molecule will be gained for each incremental C2 unit in fatty acid synthesis. For newly synthesized palmitate consisting of seven incremental C2 units, 22 deuterium atoms are incorporated in vivo in rat liver per molecule of palmitate (Lee *et al*, 1994b) and 21 are incorporated in plasma TG palmitate (Lee *et al*, 1994a).

Palmitate is the most abundant FA synthesized in the cytosol and is therefore believed to be the major primer for longer chain FA synthesis. Therefore, the newly synthesized longer chain FA molecules will be a mixture of two isotopomer populations, one having labeled and the other unlabeled palmitate as the primer (Ajie *et al*, 1995). The fractional enrichment of newly synthesized FA will be a function of the relative contribution of longer chain FAs synthesized from labeled and unlabeled palmitate, their corresponding incorporation numbers, and the enrichment in water (Ajie *et al*, 1995). Hence, stearate synthesis will result in two molecular species with either 24 (21 + 3 for complete synthesis) or 3 (for elongation of preformed palmitate) deuterium atoms per molecule (Lee *et al*, 1994a).

In order to calculate deuterium incorporation into triglyceride, average incorporation had to be determined. Jungas (1968) found that 0.87 g-atoms ^3H per g-atom C was incorporated in adipose fatty acid during fatty acid synthesis. In the VLDL fraction, the ratio of carbon to hydrogen for an average triglyceride molecule (3 monounsaturated fatty acids with 17 carbons)(Layne *et al*, 1996) can be used and corrected for glycerol carbon atoms to estimate maximum deuterium enrichment. The ratio between actual VLDL-TG enrichment and the maximum enrichment represents the relative amount of de novo synthesized fatty acid in the VLDL-TG pool (DNFAr) (Konrad, 1998).

There are three assumptions commonly made when using this method to estimate ^2H:H atoms in newly synthesized VLDL-TG FAs. These are that 1) all cell membranes are permeable to ^2H_2O, therefore deuterium enrichment is equal across pools and can be measured in plasma, 2) a consistent fraction of deuterium atoms are incorporated into FAs under various metabolic conditions, and 3) the VLDL-TG pool size remains constant over the time period studied (Leitch & Jones, 1991). The last assumption was not required for the purposes of this research, since the plasma VLDL-TG pool size is actually measured by quantitative gas-liquid chromatography.

Deuterium-labeled water has many advantages over other metabolic tracers, including being relatively inexpensive, fast equilibration with the total body water pool and requires no intravenous infusion and is therefore easily administered (Murphy, 2006). Exchange of ^2H_2O into body water pools is extremely rapid, in rodents administered an i.p. injection, plateau enrichment was reached in blood in less than 10 min and ^2H_2O enrichment was very stable over hours (Turner *et al*, 2003). Also, isotope ratio mass spectrometry is a more sensitive and reliable method than other mass spectrometric techniques. Research done using deuterium incorporation has shown that, on average, healthy males consuming 32-40% kilocalories from fat result in approximately 7-8% of TG FA or 2 grams per day as FAs (0.8 – 3.1g/d) newly synthesized from carbohydrate (Jones, 1996).

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CHAPTER 2

RATIONALE, OBJECTIVES AND HYPOTHESES

2.1 OBJECTIVES

The purpose of this research is to examine how dietary carbohydrate and fat composition may contribute to the atherogenic hypertriglyceridemia that occurs in diabetes. The objectives are to alter the carbohydrate and fat intake in subjects with diabetes versus non-diabetic subjects and examine the effect on:

1) Fasting and postprandial plasma triglyceride levels and rate of hepatic *de novo* lipogenesis,

2) The composition of fatty acids present in plasma (non-esterified) and in VLDL-TG,

3) The types of fatty acids synthesized by the liver and their contribution to VLDL-TG fatty acid composition, and

4) VLDL-TG fatty acid composition as compared to plasma free FA and dietary FA composition

Specifically, it will be determined:

1) If diabetes results in higher plasma TG levels by increasing hepatic de novo lipogenesis,

2) If higher carbohydrate intake elevates plasma TG levels by increasing hepatic de novo lipogenesis, in both diabetic and matching non-diabetic subjects

3) If increasing intake of monounsaturated fat and reducing carbohydrate aids in lowering plasma TG levels by decreasing hepatic de novo lipogenesis, while not adversely adversely affecting weight or LDL-cholesterol levels,

4) If the types of fatty acids synthesized by the liver (i.e. saturated vs. unsaturated) vary depending on the carbohydrate and fat content of the diet,

5) If VLDL-TG fatty acid composition varies depending on the carbohydrate and fat composition of the diet, and if there is an effect of diabetes,

6) If de novo lipogenesis contributes to the non-esterified fatty acids present in plasma and to what extent this may influence the VLDL triglyceride fatty acid composition.

2.2 RATIONALE

The goal of diabetes management is to prevent complications arising from the disease. In regards to dyslipidemia, the main goal is to reduce the risk of cardiovascular disease, since diabetic patients have a 3-5 fold greater risk than non-diabetic individuals with similar LDL cholesterol levels.

2.2.1 THE SIGNIFICANCE OF HYPERTRIGLYCERIDEMIA

The most common dyslipidemia in type 2 diabetes is hypertriglyceridemia; an independent risk factor for atherosclerosis and cardiovascular disease. Elevated plasma TGs have been associated with diabetes, insulin resistance, central obesity and lipoprotein abnormalities (low HDL cholesterol, small, dense LDL particles). Thus, altering TG metabolism has implications in the prevention and treatment of obesity, diabetes and cardiovascular diseases.

Recent research focuses on the postprandial state due to the atherogenic nature of postprandial lipemia and specifically the remnants of TG-rich lipoprotein particles. Because individuals are in the "fed state" for most of a 24h period, assessment of how the body handles dietary fat following a meal is fundamental to understanding the development and prevention of atherogenesis in individuals with type 2 diabetes.

2.2.2 THE EFFECT OF DIET

It has been suggested by the Canadian Diabetes Association that individuals with diabetes replace calories from saturated fat with either monounsaturated fat or carbohydrate (Canadian Diabetes Association & Clinical Practice Guideline Expert Committee, 2003). Yet, high carbohydrate intake has the potential to aggravate glucose homeostasis and the resulting increase in plasma TG has been associated with other metabolic abnormalities. High carbohydrate intake has consistently been shown to stimulate hepatic VLDL-TG synthesis and secretion, leading to increases in fasting and postprandial TG-rich lipoprotein concentration, particularly in the presence of insulin resistance. Given the evidence that low-fat/high-carbohydrate diets do not modify the basic defect in insulin resistance and seem to accentuate the metabolic manifestations, there seems to be little rationale for substituting dietary saturated fat with carbohydrate (Reaven, 2005).

Diet affects plasma TG levels and lipogenic gene expression and may be a key factor in altering TG metabolism. Dietary manipulations can play a powerful role in modulating both lipid and carbohydrate metabolism, and understanding the effect of various interventions can either exacerbate or alleviate a disease state such as insulin resistance and diabetes.

Most of the evidence supports the notion that lipogenesis is inhibited if >30% of energy from the diet is derived from fat, and carbohydrate-induction is prevented at levels of dietary fat >30% of energy. Therefore, a high carbohydrate/low fat diet formulated to have <25% energy from fat and a higher fat diet ≥35% energy from fat achieved by addition of vegetable oil should demonstrate the effect of higher amounts of dietary fat on lipogenesis. The fat level should not need to exceed 35-38% of energy to maximally inhibit fatty acid synthesis from carbohydrate. The resulting change in the balance between FA synthesis, FAs fed and FAs cleared/oxidized can be expected to alter the composition of TG in the VLDL.

2.2.3 THE CONTROVERSY SURROUNDING LIPOGENESIS

Previously it was postulated that de novo lipogenesis was a quantitatively minor pathway in humans and was not linked to carbohydrate metabolism as closely as first hypothesized. This relationship, however, remains incompletely explored. Firstly, recent research indicated that de novo lipogenesis increased to up to 20% following a meal. Fluctuations in the amount of fat synthesized may contribute to fat stored in the liver and eventually be secreted into VLDL. Because the effects of chronically increased de novo lipogenesis are not easily quantified on a physiological basis, this relationship needs to be examined. On an entirely empirical basis, a net energy surplus of 10-20 kcal per day is the equivalent of 1-2 g/day of newly synthesized FA and would be sufficient to result in a weight gain of 10-20 lbs of fat over a decade. Therefore, what appears to be a "quantitatively minor" contribution may not only have effects on lipid metabolism, but may contribute a significant quantity over the long term.

It has also been suggested that there may be regulatory functions of the de novo lipogenesis pathway (Hellerstein, 2001). The example put forth by Hellerstein is malonyl-CoA, the first committed metabolite in the de novo lipogenesis pathway. Malonyl-CoA has several known regulatory actions such as antiketogenic actions in liver, fuel selection in muscle, fuel sensing in the brain and pancreatic ß cell, and an influence on insulin secretion. Indeed, early research in this lab demonstrated that in lean versus obese mice, stimulation with an oral glucose meal is coincident with an increase in specific insulin binding to the hepatocyte nucleus and expression of genes for malic enzyme and glyceraldehyde-3-phosphate dehydrogenase within 15 minutes (Gletsu *et al*, 1999). The nuclear insulin receptor increased binding between transcription factors and the insulin receptor substrate within the malic enzyme promoter compared with that found in basal state. These findings indicate how insulin signaling may directly affect transcription of genes that control lipogenesis in the liver in response to dietary change in carbohydrate to fat intake.

It is still unclear as to whether de novo lipogenesis is involved in the pathogenesis of human disease. It seems to be associated with several syndromes, including hypertriglyceridemia, hypercholesterolemia and insulin resistance. Is this association somehow causal? It may be that de novo lipogenesis influences intracellular signaling pathways involving myristoylation, palmitoylation, or membrane fatty acids (Hellerstein, 2001).

In the case of fatty acid balance, it is difficult to define what makes a "quantitative" difference. Some fatty acids need to change only a small amount to have physiological significance and this is difficult to quantitate. However, if it is not the quantitative aspect of de novo lipogenesis that affects plasma triglyceride levels, two options remain. The process of lipogenesis could affect cellular regulation of metabolism, or an aspect of the product of lipogenesis (the qualitative aspect) may contribute to regulation in some way. It is known that the fatty acid synthesized by mammalian FAS is palmitate, however it is not known how much the production of this FA and the elongation and desaturation products vary between individuals or diets. Interindividual differences in amount of FA synthesis varies greatly, perhaps the composition of these FAs also differs enough to be distinctly important.

2.2.4 THE IMPORTANCE OF FATTY ACID COMPOSITION

The majority of research on the individual fatty acids synthesized de novo from carbohydrate has been done in animal models, and even this has been sparse and focused on development of mathematical models for isotope methods. In vivo human research has not been done extensively regarding the specific fatty acids and amounts of each produced by de novo lipogenesis. Much more research has been done surrounding the effect of dietary fat composition on the fatty acid composition of various compartments of the body. Fatty acid composition of VLDL-TG likely varies as a result of the different turnover time and selective handling of different fatty acids. Fatty acids seem to be utilized/ oxidized based on their saturated profile and/or chain length (DeLany *et al*, 2000;Jones *et al*, 1985). Oleic acid and linolenic acid seem to be oxidized rapidly, whereas palmitate and stearate are oxidized more slowly (DeLany *et al*, 2000).

Early research in this lab established that normal, physiological changes in diet fat intake alter fatty acid composition of the adipocyte membrane (Clandinin *et al*, 1996;Field *et al*, 1990) and skeletal muscle membrane phospholipids (Liu *et al*, 1994). Increased membrane polyunsaturated fatty acid improved insulin responsiveness (Field *et al*, 1990) by increasing insulin stimulated glucose transport and net protein synthesis and also altering the phosphorylation state of the receptor (Gletsu & Clandinin, 1997). It has since been well accepted that fatty acid profile of the diet affects the fatty acid profile of many tissues of the body.

Interestingly, recent research has indicated that, as opposed to varying fat composition, varying total fat intake can also affect the composition of fatty acids present in serum phospholipids, TG, cholesteryl esters and free fatty acids. It seems that the proportion of saturated fatty acids increases and linoleic acid decreases in these serum lipids following a low fat diet (Raatz *et al*, 2001). This has been partially attributed to the contribution of endogenously produced FAs following high carbohydrate/low fat intake. If TG produced by the liver changes the composition of VLDL-TG to a more saturated profile, this could have metabolic consequences. Fatty acids hydrolyzed from VLDL-TG are available throughout the body in both fasting and feeding states. A higher VLDL saturated fat content could result in a more saturated cell membrane composition in either adipose or muscle tissue and have important physiological effects on insulin action. At the cellular level, for instance, this could be one factor contributing to insulin resistance (Hellerstein, 1999). Indeed, serum fatty acid composition has been shown to predict the long-term development of the metabolic syndrome (Warensjo *et al*, 2005).

If feeding varying amounts of fatty acids results in different proportions of fatty acids in the various body compartments, it follows that unique changes may also occur with carbohydrate feeding. The availability and exposure of the body to high amounts of fat, particularly saturated fat, following a high fat diet has long been suspected in the pathology of many diseases such as obesity and diabetes. However, within the range of fat normally consumed by humans, it is possible that the amount of fat and resulting plasma levels do not vary substantially between diets. This is especially true when considering the evidence that a mixed meal high in carbohydrate has the potential to result in higher postprandial TGs than that of a higher fat/lower carbohydrate meal.

It is known that the fatty acid profile of the diet (i.e. chylomicron TG) has an effect on lipid and lipoprotein metabolism. It is not known whether the fatty acid profile of the other sources of fatty acid in the plasma (FFAs, VLDL-TG) differ and therefore may be a cause or consequence of diet and/or diabetes. Certainly, availability of fat in the liver may be the main driver of VLDL overproduction (Adiels *et al*, 2006;Sakata *et al*, 1993) and reducing the availability of saturated fatty acids may be key. Because insulin is important in the inhibition of VLDL secretion (Lewis *et al*, 1995;Malmstrom *et al*, 1997), a high carbohydrate diet may not be the most beneficial to insulin resistant states. The resulting potential to aggravate hyperglycemia in combination with hyperinsulinemia increases or fails to affect the sources of TG for VLDL production. In insulin resistance, a high carbohydrate meal may increase plasma insulin levels resulting in increased lipogenesis and the availability of saturated fatty acids for VLDL production. At the same time, high insulin no longer suppresses VLDL secretion or free FA release into plasma and these add to the chylomicrons already present in high amounts in the blood, overwhelming clearance mechanisms. In this way, reducing lipogenesis may be an important factor in reducing plasma TG levels. It has also been shown that the type of lipid available may have an effect on VLDL secretion, as in vitro exposure of rat hepatocytes to fish oil increased apoB degradation (versus oleic acid) (Brown *et al*, 1997). Therefore one could conjecture that if de novo lipogenesis changes the lipid composition of the hepatocyte or cytosolic lipids, this might affect VLDL synthesis and secretion.

2.2.5 RECENT TECHNOLOGICAL ADVANCES

Isotope ratio mass spectrometry utilizing stable isotopes is one of the few ways that de novo lipogenesis can be directly and reliably measured in human subjects. The methods in Experiment 2 employ the use of a gas chromatograph pyrolysis mass spectrometer (GC/P/IRMS), a relatively new tool in human metabolic research. The methodology surrounding this instrument in the area of lipid research is so new that it has required some development. Unlike animal studies that employ various tracers for measuring specific fatty acids synthesized de novo, or using mass spectrometry and deuterium incorporation techniques to measure total fatty acid synthesis, this instrument is able to measure the deuterium incorporation into specific fatty acids using the same simple, relatively inexpensive, non-invasive procedures as traditional deuterium incorporation techniques.

2.2.6 SIGNIFICANCE OF RESEARCH

There is presently a need for well-designed studies analyzing the effect of diet on diabetic and insulin resistant states involving free-living human subjects. To date, much of the research designed to assess the contribution of de novo lipogenesis to hypertriglyceridemia examines only the fasting state and excludes subjects with insulin resistance, type 2 diabetes and/or family history. Few *in vivo* human studies have investigated the FAs synthesized and cleared, and none to date have investigated insulin resistant or diabetic subjects. Findings from this research will contribute to understanding the difference in FAs in postprandial versus fasting VLDL-TG, defining differences in FA composition and sources assembled into VLDL particles. Postprandial measures will establish individual FAs that are newly synthesized (i.e. rapidly labeled with deuterium as opposed to synthesized with endogenous NEFA) and most rapidly cleared.

More than two million Canadians have diabetes and this number is expected to increase dramatically as the population ages and becomes more obese. Cardiovascular disease is the leading cause of morbidity and mortality in individuals with type 2 diabetes. Understanding the mechanisms underlying elevated TG levels in diabetic dyslipidemia is critical to decreasing risk. One contributing factor to plasma TG is de novo lipogenesis, although the role is poorly understood. The synthesis of individual fatty acids and the conversion of dietary palmitate to other fatty acids may differ depending on diet and metabolic state. This research will result in new information and insights into fully understanding the pathology of altered lipid metabolism in diabetes and the role of diet, as well as in determining preventative strategies.

2.3 HYPOTHESES

2.3.1 EXPERIMENT 1 (CHAPTER 3)

Lowering the carbohydrate intake by replacement of those calories with monounsaturated fat will:

1) Reduce plasma TG levels and hepatic de novo lipogenesis,
2) Have a greater effect in subjects with higher baseline serum TG levels,
3) Have a similar effect in matched diabetic subjects, and
4) Not adversely affect weight or cholesterol levels.

2.3.2 EXPERIMENT 2 (CHAPTERS 4, 5 AND 6)

Additionally, it is hypothesized that:

1) Diabetes and/or lower fat intake will result in higher fasting and postprandial plasma triglycerides and that these differences will be reflected in VLDL-triglyceride levels.
    - It is specifically hypothesized that a higher composition of palmitate and stearate and lower composition of oleate will characterize the fatty acid composition differences between diabetes/lower fat intake and non-diabetes/high oleate intake, perhaps due to effects of fatty acid composition on production or clearance of VLDL-triglyceride.
2) De novo synthesis of total and saturated fatty acids is higher in diabetic subjects and also after lower fat/higher carbohydrate intake, resulting in differences in VLDL-triglyceride fatty acid composition.
3) Plasma free fatty acid composition is similar to VLDL-triglyceride fatty acid composition as these are the main source of fatty acids for VLDL-triglyceride synthesis.
    - There may be a small contribution of 'extra-hepatic' de novo fatty acid to VLDL-TG synthesis, and this will be detected in plasma free fatty acids through the release of labeled fatty acids from adipose tissue triglyceride lipolysis (fasting) or spillover from chylomicron triglyceride lipolysis (postprandial).

Overall, it is expected that a contributing mechanism underlying alterations in TG metabolism during high carbohydrate intake or in insulin resistance may differ from that resulting from high oleate/higher fat diets. Reducing carbohydrate and raising monounsaturated fat intake should reduce plasma TG and VLDL-TG levels, decrease endogenous synthesis of TG and change VLDL-TG composition resulting in increased clearance. If diabetes and/or insulin resistance alters postprandial TG amount and composition, this alteration has not been definitively characterized. Higher glucose/insulin levels following higher carbohydrate intake may promote VLDL-TG composition high in palmitate and stearate from de novo lipogenesis. Higher plasma TG values observed in the diabetic state or when consuming a high carbohydrate diet may occur as a consequence of synthesis of saturated fatty acids that are less readily cleared from the VLDL. This could lead to perturbations of the postprandial state and result in inflammation.

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CHAPTER 3

HYPOTRIGLYCERIDEMIC EFFECT OF HIGH MONOUNSATURATED FAT INTAKE IN FREE-LIVING HUMAN SUBJECTS WITH TYPE 2 DIABETES.

3.1 INTRODUCTION

The effect of diet on de novo lipogenesis in humans and the relationship to plasma triglyceride (TG) levels remains a controversial area that requires investigation. Diet is known to affect TG metabolism, however the relationship is complex and little is known about how fat in the diet reduces fat in the blood. Further, the effect of dietary fat amount and composition on TG metabolism in free-living humans has only recently been explored using reliable stable isotope methods in vivo. It is important to study this phenomenon in type 2 diabetes given that altered carbohydrate and lipid metabolism play key roles in determining plasma TG concentration and the pathogenesis of atherosclerosis.

The atherogenic lipid profile that is central to the metabolic syndrome and type 2 diabetes, predisposing these conditions to cardiovascular disease, is characterized by hypertriglyceridemia and low HDL cholesterol levels. Elevated fasting plasma TG concentration has been identified as a cardiovascular disease (CVD) risk factor, independent of HDL cholesterol (Austin *et al*, 1998;Cullen, 2000;Tanne *et al*, 2001). In patients with type 2 diabetes, hypertriglyceridemia is an independent risk factor for atherosclerosis (Temelkova-Kurktschiev *et al*, 1997).

A high carbohydrate/low fat diet has traditionally been recommended for the prevention of atherosclerosis in diabetes patients due to the beneficial effect on weight reduction and LDL cholesterol concentrations. However, some higher carbohydrate diets tend to decrease HDL cholesterol and increase plasma TG concentrations. This elevation in TG is known as "carbohydrate-induced hypertriglyceridemia," (reviewed by Parks & Hellerstein, 2000) and may last as long as the diet is fed in healthy subjects (Knopp *et al*, 2000). Carbohydrate induction seems to be particularly persistent in patients with type 2 diabetes (Coulston *et al*, 1989;Garg *et al*, 1994). Differences in the hypertriglyceridemic effect of the type of carbohydrate fed (i.e. fructose vs. glucose vs. starch) may be less pronounced with higher fat diets consisting of up to 30% of energy as fat (Herzberg, 1991;Jeffcoat & James, 1977). Furthermore, higher monounsaturated fatty acid (MUFA) diets have been shown to improve many other CVD risk factors, including blood glucose control (Garg *et al*, 1988;Rasmussen *et al*, 1993), blood pressure (Rasmussen *et al*, 1993;Appel *et al*, 2005) and lipid profiles (Rivellese *et al*, 1994;Garg, 1998;Appel *et al*, 2005) when compared to lower fat/higher carbohydrate diets. The mechanism by which a higher MUFA diet exerts beneficial effects is likely multifactorial.

Initially it was hypothesized that carbohydrate-induced hypertriglyceridemia resulted from increased production of VLDL-TG through conversion of carbohydrate to fat by hepatic *de novo* lipogenesis (Parks & Hellerstein, 2000). Research has shown that de novo lipogenesis may play a role in the pathogenesis of hypertriglyceridemia (Sebokova *et al*, 1996), however the quantitative contribution of lipogenesis to plasma TG concentration in healthy adult humans has previously been considered minor (Hellerstein, 1999). Although the contribution of de novo lipogenesis to VLDL-TG may be quantitatively less in humans than in animals, there is potential for a substantial increase of up to 10 fold over the short term with carbohydrate overfeeding (Acheson *et al*, 1988). Several studies, including previous research in this lab, indicated that in healthy subjects there is a relationship between plasma TG concentration and hepatic TGFA synthesis (Hudgins, 2000;Konrad *et al*, 1998). There is also evidence that some subjects may have a threshold at normal carbohydrate intakes (Knopp *et al*, 2000). Because the effects of chronically elevated de novo lipogenesis are not easily quantified on a physiological basis, this relationship needs to be explored. On an entirely empirical basis, a net energy surplus of 10-20 kcal per day is the equivalent of 1-2 g/day of newly synthesized FA and would be sufficient to result in a weight gain of 10-20 lbs of fat over a decade. Therefore, what appears to be a "quantitatively minor" contribution may not only have effects on lipid metabolism, but may contribute a significant quantity over the long term.

In previous studies examining de novo lipogenesis, respondents with diabetes or a family history of the disease have been excluded. Many of these studies have also involved either liquid meals or highly controlled metabolic ward situations. Therefore, one of the aims of this study was to examine the effect a small change in diet over the longer term in free-living patients with diabetes versus a group of comparable non-diabetic individuals.

The purpose of this study was to determine if low fat/high carbohydrate intake contributes to elevated plasma TG concentrations in free-living type 2 diabetic subjects and if increasing MUFA and decreasing carbohydrate intake aids in reducing TG concentrations. We hypothesized that isocaloric replacement of carbohydrate with olive oil, in the context of the usual diet of free-living subjects with diabetes, would result in lower plasma TG concentrations due to a reduction in hepatic VLDL-TGFA synthesis. We further hypothesized that this effect would be greater and more clinically relevant in subjects with higher plasma TG concentrations.

3.2 SUBJECTS AND METHODS

3.2.1 STUDY PARTICIPANTS

The Faculty of Agriculture, Forestry and Home Economics Human Ethics committee approved the procedures followed in this study. Volunteers were recruited by contacts made through the outpatient diabetes clinic at the University of Alberta Hospitals and advertisements posted at the University of Alberta. All respondents gave written informed consent before enrolling in the study and were screened for chronic disease, smoking, medications, supplements, family history of diabetes and cardiovascular disease, fasting plasma lipids, reasonable diabetes metabolic control, and dietary macronutrient composition. Respondents with type 2 diabetes (diagnosed as having diabetes for >3y) were matched for age, sex and weight with non-diabetes subjects. Baseline measures to verify metabolic status included plasma concentrations of lipids, fasting glucose, creatinine, C-peptide, ALT, alkaline phosphatase, HbA_{1C} and insulin. Respondents having plasma TG concentrations > 3.0 mmol/L were referred for treatment and those using medications known to alter lipid metabolism were not eligible for inclusion in the study. Macronutrient intake of eligible participants was estimated using 7-day food records and Food Processor II nutrient analysis software (V7.4, Esha Research). Thirty subjects were preferentially selected on the basis of fat intake level (≤35% of energy from fat) so that the lower fat intake period would most closely resemble the subjects' usual dietary intake. Of the eligible participants who entered the study, 2 dropped out before completion of the study design.

3.2.2 STUDY DESIGN

Subjects served as their own control in a crossover design where each diet period consisted of 3 months. All subjects were free-living and were instructed not to change their medications or dose, usual exercise or basic dietary routine throughout the study. Subjects were counseled by a registered dietitian to achieve a higher carbohydrate/lower fat intake (LF) or a lower carbohydrate/higher fat intake (HF). This was based on 'usual' diet according to 7d food records provided at study entry and following each diet period. The HF intervention was achieved by addition of olive oil (as visible oil and spread) to each subject's usual diet, while decreasing consumption of the higher carbohydrate foods identified in food records (i.e. bread, cereal and pasta). The 7d food records provided at the end of each diet period comprised the last week of each period, at which point the dietitian verified food record items, weight/portion size, compliance and explained the instructions for the next diet period. The intended dietary change was to result in an increase in fat intake, with a concomitant isocaloric decrease in carbohydrate consumption in the HF diet period (versus the LF diet). If subjects did not achieve the dietary design, the opportunity to complete another diet period was provided. Several subjects repeated diet periods (*n*=4).

On the second last day (background day) of each diet period, a 25 mL fasting blood sample was taken by venipuncture into vacutainer tubes containing EDTA and in tubes containing no EDTA for collection of 5 mL serum. Subjects consumed a priming dose of deuterium oxide (99.9 atom % Deuterium, C/D/N Isotopes) at 0.5 g ^2H_2O/kg estimated body water (60% of body weight). A maintenance dose of 1.0 g ^2H_2O/kg estimated body water was provided in a 2L bottle of drinking water to be consumed at regular intervals over the next 24h to compensate for unlabeled water obtained in the diet. Deuterium incorporation into body water rapidly reaches plateau and is maintained at steady state for many days due to the slow turnover of body water in humans (Turner *et al*, 2003). On test day 2 (enrichment day) a second 25 mL fasting blood sample was drawn 24h after the background samples.

3.2.3 ANALYTICAL METHODS

Serum samples were sent to the local clinical laboratory for determination of fasting plasma insulin, cholesterol (total, LDL, HDL), and TG concentrations. Glucose and HbA_{1C} were analyzed on diabetes group samples. Cholesterol and TG concentrations were determined by enzymatic assays. Hepatic TG fatty acid synthesis was determined by the deuterium incorporation method (Leitch & Jones, 1991). Samples used for calculation of TGFA synthesis were centrifuged at 3000 rpm x g for 15 minutes at 4 ºC to obtain plasma (Jouan refrigerated centrifuge, CR 4.11). Plasma VLDL was isolated and TG fraction purified as described (Layne *et al*, 1996). Briefly, deuterium enrichment in VLDL-TG and plasma water was measured in duplicate samples from each subject before and after 24h of oral administration of ^2H_2O. Samples were reduced to hydrogen gas and the deuterium to hydrogen ratio was analyzed by isotope ratio mass spectrometry (Finnigan MAT, Germany) and used to measure deuterium enrichment of the VLDL-TGFA. Calculation of TGFA fractional synthetic rate (FSR) was done as previously described and from this the relative amount of *de novo* fatty acid or relative synthesis of VLDL-TGFA (DNFAr) was estimated (Konrad *et al*, 1998).

3.2.4 STATISTICAL METHODS

A power analysis using data from a previous study (Konrad *et al*, 1998) was performed to enable detection of a 20% decrease in plasma TG concentration at 95% power to detect a difference between diets. Estimation of sample size from this calculation resulted in a requirement of a minimum 8 subjects to complete the study design.

Data was analyzed to evaluate the effect of diet and diabetes on outcome measures and then as two groups (diabetes and control group). Triglyceride data was normally distributed as verified by S-Plus 6.1, however, one subject was statistically an outlier for the TGFA FSR data (control subjects, *n*=1). The data points for this subject were left in all analyses due to the interindividual variation inherent in FSR data. All statistical analyses of outcome measures were performed using SAS (V8.0; SAS Institute Inc.). Correlation between TGFA synthesis and fasting plasma TG concentration was done using PROC CORR. One-way analysis of variance (ANOVA) was used to test the effect of diet order and gender. Two-way ANOVA was used to test the effect of diet and diabetes on plasma TG, LDL and HDL cholesterol concentrations and VLDL-TGFA FSR and one-tailed paired comparisons were used to test changes in these parameters following LF and HF intake within groups. To evaluate the effect of diet and diabetes in subjects with higher TG, the groups were split based on median TG concentration in the LF period and compared. All data is illustrated as mean ± SD. Probability values <0.05 were considered significant.

3.3 RESULTS

3.3.1 DIETARY ANALYSES

Of the total 28 subjects, 4 did not achieve fat intake of <35% of energy in any diet period (not qualifying as "lower" fat) and 5 subjects did not achieve a 5% increase in fat energy or higher in the HF diet period. Therefore, a total of 19 (10 control, 9 diabetes) subjects met the study design and were included in the results.

As per diet counseling, subjects had increased total fat and MUFA intake while decreasing total carbohydrate intake. Dietary fat, carbohydrate and protein data from 7-day food record analyses were tested for significant differences on a percentage of energy basis. No significant differences were detected between diet periods except for carbohydrate and fat, including both SFA and MUFA (P=<0.0001). There were no differences between the diet periods of the control and diabetes groups.

Table 3-1. Diet composition of free-living subjects as assessed by 7-day food records in the final week of each diet period.

	Diabetes		Control	
	LF	HF	LF	HF
Energy *kcal*	1689 ± 445	1924 ± 482	1698 ± 428	1703 ± 421
Protein	19 ± 3	18 ± 3	16 ± 3	17 ± 3
Carbohydrate	55 ± 3	50 ± 5	57 ± 4	49 ± 4
Fiber *g*	23 ± 8	25 ± 7	19 ± 6	20 ± 8
Sugar *g*	86 ± 26	87 ± 31	104 ± 40	84 ± 31
Fat	27 ± 10	33 ± 10	27 ± 8	35 ± 8
SFA	8 ± 2	11 ± 3	8 ± 3	11 ± 2
MUFA	9 ± 4	13 ± 4	9 ± 3	14 ± 5
PUFA	5 ± 4	5 ± 3	4 ± 2	5 ± 1
PUFA:SFA	0.65 ± 0.28	0.46 ± 0.15	0.52 ± 0.11	0.48 ± 0.11
Cholesterol *mg*	257 ± 93	269 ± 75	178 ± 69	233 ± 67

Mean ± SD. Values are % energy unless otherwise indicated. LF: lower fat intake; HF: higher fat intake, CHO: carbohydrate, SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

3.3.2 CLINICAL MEASURES

There were no differences between control and diabetes subject groups (Table 3-2), nor between LF and HF periods for any characteristic.

Table 3-2. Subject characteristics.

	Control	Diabetes
N *m/f*	3/7	4/5
Age *y*	52 ± 9	59 ± 10
BMI kg/m^2	29 ± 8	30 ± 5
Glucose *mmol/L*	5.2 ± 0.6	6.6 ± 0.5
Hb$_{A1c}$%	5.4 ± 0.2	6.1 ± 0.3
Triglycerides *mmol/L*	1.22 ± 0.6	1.28 ± 0.4
LDL cholesterol *mmol/L*	3.27 ± 0.6	3.13 ± 0.7
HDL cholesterol *mmol/L*	1.62 ± 0.4	1.41 ± 0.3
Weight after LF *kg*	80.2 ± 25	87.4 ± 16
Weight after HF *kg*	79.8 ± 25	88.1 ± 17

Mean ± SD. LF: lower fat intake, HF: higher fat intake.

There was no effect of diet order, gender or diabetes on fasting plasma TG or LDL-c concentration. There was an effect of gender on plasma HDL-c levels; male subjects had a lower mean HDL-c concentration than female subjects for both diet periods (P=0.03, data not shown). For the diabetes subject groups, glucose and HbA_{1C} concentrations were not different between diet periods.

The plasma TG concentration following each diet period for each subject is shown (Figure 1). Data was analyzed as one group for the effect of diet and disease on TG concentration. There was no overall effect of diabetes but there was a significant effect of diet on TG concentration. When analyzed separately as control and diabetes subject groups, there was a trend toward a lower fasting plasma TG concentration after HF intake when compared to LF (diabetes: -12%, P=0.065; control: -14%, P=0.053). There were no significant differences found in TG level between the two groups (Figure 2).

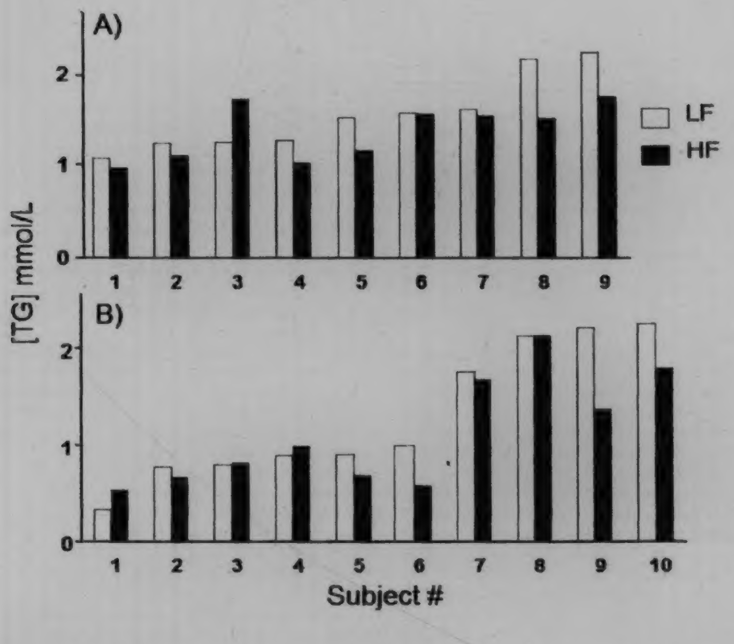

Figure 3-1. The effect of lower fat and higher fat intake on fasting plasma triglyceride concentration in individual subjects. A) Diabetes subjects and B) control subjects ordered from lowest to highest plasma TG concentration following the lower fat (LF) diet phase. Higher fat (HF) values are also indicated.

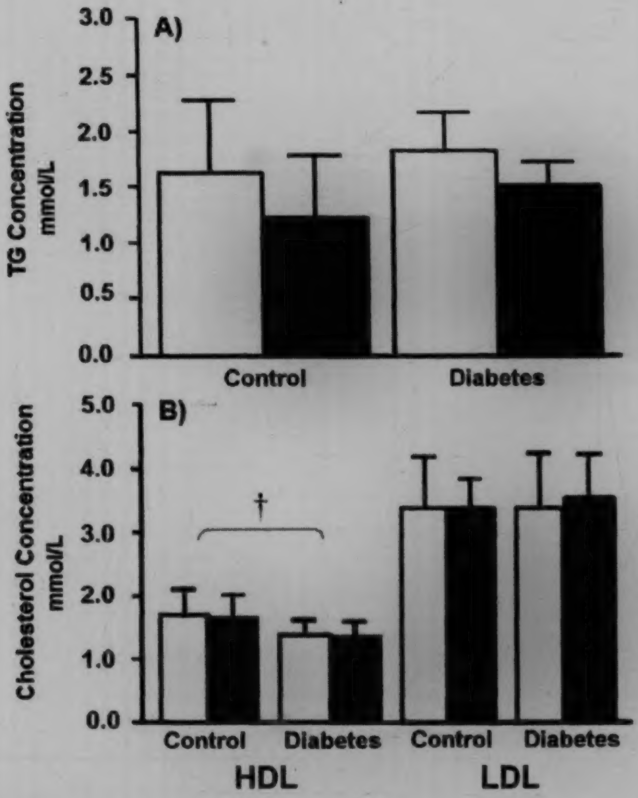

Figure 3-2. The effect of lower and higher fat intake on fasting plasma lipids in diabetes and control groups. Mean A) triglyceride and B) HDL and LDL cholesterol concentrations. Bars represent mean value following a 3-month period of lower fat (white) and higher fat (black) intake, error bars indicate SD. † represents a significant difference between the diabetes and control group ($p<0.01$).

It was hypothesized that TG reduction would be greater in subjects with higher TG concentrations as TG levels that are already low (Figure 1) are less likely to be clinically relevant. To test this, subgroups with higher TG were evaluated based on median TG concentration following the LF intake period (control group: 0.95 mmol/L, diabetes group: 1.53 mmol/L). There was a significantly lower plasma TG following the HF intake for both control and diabetes subjects in the higher TG subgroups (-19.4%, 1.88 ± 0.5 vs. 1.51 ± 0.6 mmol/L, P=0.04 and -17.3%, 1.83 ± 0.3 vs. 1.51 ± 0.2 mmol/L, P=0.03, respectively. Data not shown).

There were no significant changes in LDL or HDL cholesterol concentrations between diet periods in either group (Figure 2b) except that the diabetes group as a whole had a lower HDL-c concentration than the control group (1.36 vs. 1.67 mmol/L, P=0.007).

3.3.3 DE NOVO LIPOGENESIS

There was no effect of diet order, gender or diabetes on fasting on VLDL-TGFA synthesis. The range of estimates for the fraction of de novo synthesized fatty acid in the VLDL-TG pool ($DNFA_r$) was 1.17-26.27% and 0.58-9.63% and the pool of TGFA synthesized was 0.20-5.36 g and 0.17-2.83 g in control and diabetes subjects, respectively. For all subjects together, there was no effect of diet or diabetes on hepatic TGFA FSR and no significant differences between control and diabetes groups found, however, within groups there was an effect of diet. The control group exhibited a 34.5% higher TGFA FSR in the HF than the LF diet period (P=0.05, DNFAr of 7.84% vs. 5.13%, respectively; Figure 3-3). When the higher triglyceride subgroups were compared, no effects of diet on FSR or DNFAr were found.

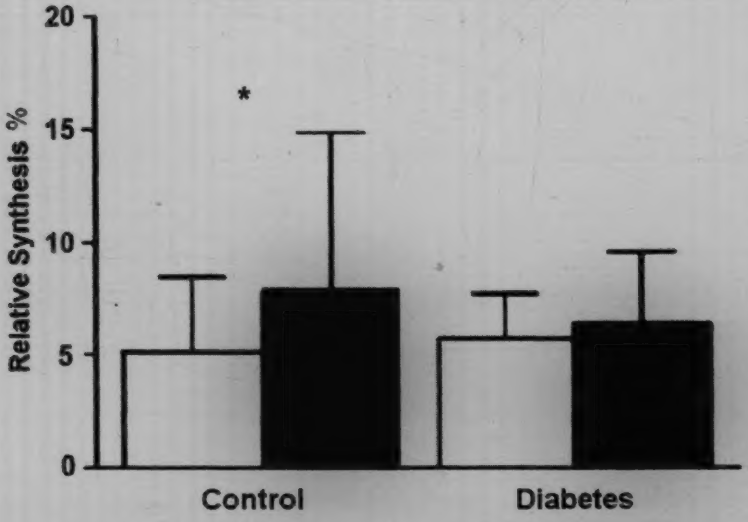

Figure 3-3. The effect of lower and higher fat intake on VLDL-triglyceride fatty acid fractional synthetic rate in diabetes and control groups.
Bars represent the mean value following a 3-month period of lower fat (white) and higher fat (black) intake. Error bars indicate SD and * indicates a significant difference between VLDL-TGFA FSR (fractional synthetic rate) following the higher fat diet than the lower fat diet in the control group (p=0.05).

There were no correlations found between change in TG and change in FSR from a LF to HF diet for either control or diabetes groups (data not shown). However, hepatic TGFA FSR was significantly correlated with plasma TG concentration following LF intake for all subjects (0.68, P=0.001, R^2=0.47; data not shown). For control subjects, TGFA FSR was highly correlated with plasma TG concentration following both LF and HF intake (Figure 4B). When the outlier was removed from this data, the relationship remained significant for the LF intake period only (R^2=0.52, P=0.03). Diabetes subjects showed no apparent correlation between hepatic TGFA synthesis and plasma TG concentration (Figure 4A).

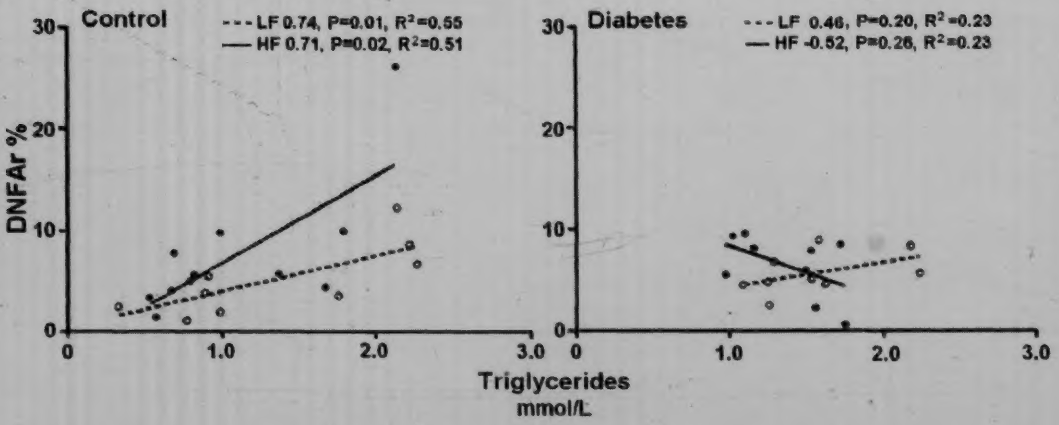

Figure 3-4. The relationship between fasting plasma triglyceride concentration and hepatic de novo triglyceride fatty acid following lower and higher fat intake.

A) Diabetic group and B) control group. Symbols represent the individual value following a 3-month period of LF (lower fat; open circle) and HF (higher fat; solid circle) intake. There was a significant relationship between TG and DNFAr in the control group as tested by two-tailed correlation. No significant relationships between TG and DNFAr were found for the diabetes group following either LF or HF intake. When the outlier in control DNFAr data was removed, the relationship between TG and FSR in control subjects remained significant following the LF period only (LF: 0.72, P=0.03; HF: 0.47, P=0.20).

3.4 DISCUSSION

3.4.1 THE EFFECT OF FAT INTAKE ON PLASMA TRIGLYCERIDES

The present study was designed to mimic the free-living situation where small changes in dietary intake occur within the scope of an individual's habitual diet. Subjects were counseled to increase their fat intake by incorporating visible olive oil into their usual diet, while isocalorically reducing carbohydrate-rich foods. Seven-day food records were used to screen for dietary fat intake change of at least 5% of energy, as it was suggested that this is the minimum required to effect a change in fasting TG when fat is replaced with carbohydrate (Parks, 2002). The exchange of carbohydrate and fat calories in this study resulted in a similar TG-lowering effect for both diabetic and non-diabetic subjects. There was trend towards a decrease in plasma TG concentrations in both control and diabetes groups after counseling to add olive oil and decrease carbohydrate in their diets. This was significant in a subgroup of subjects in both groups with higher TG when consuming lower fat but there was no relationship between change in fat intake and change in TG concentration. The overall TG-lowering effect of higher fat intake was very consistent as 14/19 subjects exhibited lower plasma TG concentrations after HF compared to LF intake (Figure 1). Three of the subjects that did not show this effect were in the control group and had plasma TG concentrations of <0.90 mmol/L. It may be important in future studies to screen participants and exclude those with fasting plasma TG at levels in which dietary treatment would not be clinically relevant.

3.4.2 MONOUNSATURATED FAT

Many studies involving healthy subjects have shown that increasing carbohydrate in the diet has a consistent TG-elevating response (Parks & Hellerstein, 2000). Previous long-term studies involving free-living subjects indicate that both a low total fat/high fibre diet and a high-unsaturated fat lower fibre diet reduce LDL cholesterol and TG concentrations, without negatively affecting HDL cholesterol, plasma glucose or insulin concentrations (Rivellese *et al*, 1994). However, it has been suggested that MUFA diets may be most beneficial when fat intake is not too high (i.e. <37% of energy) (Vessby *et al*, 2001). In healthy normolipidemic subjects who habitually consume a high complex carbohydrate/high fiber diet, it is possible that a dietary macronutrient adjustment by isocaloric exchange of carbohydrate with olive oil may not have a significant effect on TG concentrations, as the effects of both diets on lipoprotein profiles should be similar (Mensink & Katan, 1987). The TG-lowering effect of higher MUFA/lower carbohydrate intake in the current study is consistent with other research demonstrating that fatty acids lower fasting TG when substituted for carbohydrate (Grundy, 1986;Katan *et al*, 1994;Garg, 1998;Berry *et al*, 1992). These results indicate that even a small change in habitual diet by free-living diabetic subjects over several months consistently decreases plasma TG concentration.

There were a number of exclusions in this study due to insufficient change in diet. Despite the benefits of higher MUFA diets, subjects expressed concern about consuming added olive oil and were unable to increase their fat intake. Amongst type 2 diabetes patients this may be a factor (Vessby, 1995) because obesity and cardiovascular disease are major concerns in this population. Further, self-reporting of food intake has an element of error involved, particularly in the assessment of energy intake and more importantly, in the measurement of actual dietary change (Schoeller, 1995). There were a number of participants in which we could not detect a change in fat intake by analysis of 7-day food records. However, any error in the assessment of energy contributed by macronutrients and fatty acids were likely reduced by the 7-day length of the food records and by the rigorous nature of reviewing the individuals' recorded intake by the registered dietitian. The crossover design of this study also increases the meaningfulness of the results even though sample size and lack of control over dietary factors may limit generalization. There was an unintended overall increase in SFA along with increases in MUFA and total fat. Caution should be taken when counseling free-living diabetes patients to increase fat intake in order to ensure a proportional increase dietary unsaturated fat. Olive oil contains some SFA (up to 15%) and perhaps this should be considered when adding olive oil to the diet. The intake of carbohydrate-rich foods should not only be limited, but also SFA-containing foods.

3.4.3 DE NOVO LIPOGENESIS

Although the benefits of high MUFA diets are well-known, the underlying mechanisms for the effects are unclear. Research examining the effects of fatty acids on lipogenesis are mostly limited to animal studies. Contrary to our hypothesis, the changes measured in hepatic TGFA FSR did not correspond to changes in plasma TG concentration in this group of subjects. However there was a positive correlation between relative rate of fatty acid synthesis (DNFAr) and TG in control subjects regardless of diet, indicating that non-diabetic subjects with higher TG levels also have higher rates hepatic TGFA synthesis (Figure 3B). When the outlier was removed, this relationship remained significant for the lower fat intake period only. It is possible that the relationship between fasting plasma TG concentration and de novo lipogenesis is stronger for some individuals when consuming a lower fat/higher carbohydrate diet. Interestingly, this relationship did not exist for the diabetes group (Figure 3A), even though the TG response to diet change was very similar to the control group (Figure 2).

The diet change involved in this study may not have been large enough to achieve a large difference in hepatic de novo lipogenesis. Previous research has indicated that dietary energy from fat of >30% results in a reduction of not only hypertriglyceridemia, but also lipogenesis (Jeffcoat & James, 1977). However, most research showing dietary inhibition of lipogenic genes in animals pertains to polyunsaturated fat. Despite the known benefits of high MUFA diets on TG levels, the effect of a higher MUFA diet (total fat energy of 30-37%) on hepatic de novo lipogenesis was previously unknown. The current study was designed to achieve a fat intake change within the range of lower fat (~25% of kcal) to higher fat (~35% of kcal) (Table 2) in order to maximally support both an inhibition of lipogenesis and a reduction in TG concentrations. In this study, even though higher MUFA intake resulted in a trend towards lower plasma TG concentrations, the opposite trend was found for de novo lipogenesis and this requires further investigation.

It is possible that either the change in fat intake was not of the magnitude required to result in a change in lipogenesis or that some subjects did not isocalorically exchange carbohydrate and fat intake. This combined with higher energy intake by diabetic subjects may have influenced lipogenesis rates as well. Also, according to food records, as SFA and MUFA intake increased, polyunsaturated fat (PUFA) intake between the HF and LF diets remained unchanged. This may have contributed to inhibition of de novo lipogenesis at higher carbohydrate intake because SFAs and MUFAs may not inhibit lipogenesis as effectively as PUFA (Hudgins, 2000). Animal studies have shown that SFA and MUFA do not inhibit lipogenic genes whereas PUFA inhibits lipogenesis even during overfeeding a high fat/high carbohydrate diet. It is not known what effect any increase in SFAs might have on de novo lipogenesis.

Previous research using the deuterium isotope technique in healthy subjects indicated a relationship between fasting plasma TG concentration and hepatic de novo lipogenesis (Konrad *et al*, 1998). Other isotope studies in humans have shown that de novo lipogenesis has a role in plasma TG concentration (Letexier *et al*, 2003). Studies done in lean and obese subjects by Hudgins et al. showed an increase in lipogenesis as well as plasma triglyceride level after feeding a diet rich in simple sugars. This effect seemed to depend on whether there was a constant elevation in lipogenesis or a diurnal pattern to the elevation. It was also highly variable between subjects, comparable to the present study in the demonstration of high individual variation in the range of de novo lipogenesis values (Figure 2)(Hudgins, 2000).

It should be noted that in the current study, a single fasting sample was used to measure 24h FSR/DNFAr and this may not have fully captured the impact of fatty acids synthesized over 24h or the variation that may occur following a meal. Recently in a study involving healthy subjects, a temporal pattern of lipogenesis during the postprandial state was shown. Lipogenesis was stimulated after meals, peaking at approximately 4 hours, whereas triglyceride concentration peaked sooner (Timlin & Parks, 2005). This suggests a contribution of de novo lipogenesis to prolonged postprandial lipemia that is not indicated by fasting measurements. Individuals exhibiting prolonged postprandial lipemia may also have greater postmeal de novo lipogenesis contributing to a reduction in triglyceride clearance and subsequent reduction in fasting plasma triglyceride concentration. It is possible that the 24 hour FSR as measured in this study may not show a complete picture of the 24 hour rate, but may vary depending on time of measurement. Animal studies have indicated that genes involved in de novo lipogenesis can be upregulated or downregulated within hours (Clarke *et al*, 1990), and therefore de novo lipogenesis may be turned on and off within short periods by oral and hormonal influences.

Research using isotopomer distribution to follow VLDL-TG metabolism has indicated that decreased clearance underlies carbohydrate-induced hypertriglyceridemia, rather than increased synthesis (Parks *et al*, 1999). Indeed, subjects consuming low fat/high carbohydrate diets seem to have higher remnant lipoprotein concentrations (Abbasi *et al*, 2000). Notably, the current study did not 'induce' hypertriglyceridemia but measured the effects of increasing total fat and MUFA intake by normolipidemic subjects. Whether or not the mechanism involved is similar to the classically defined 'carbohydrate-induced hypertriglyceridemia' remains to be determined.

3.5 CONCLUSION

The results of this study indicate that higher fat intake achieved by increasing olive oil consumption could have beneficial effects on fasting plasma TG concentrations or borderline hypertriglyceridemia in type 2 diabetes subjects. Hypertriglyceridemic subjects may also benefit, but are usually treated pharmacologically. The free-living nature of this study lends itself to the applicability of the observations, although a number of considerations need to be made when designing future studies. Free-living subjects require close monitoring for compliance in order to maintain the sample size at a level necessary for sufficient power to detect a true difference. It is very important that the higher fat diet consumed be isoenergetic and that it is achieved by increasing unsaturated fat and not cholesterol or saturated fat. The hypotriglyceridemic effect of higher fat/high MUFA/lower carbohydrate consumption seems to be more complicated than fasting measurements would indicate. Even though a reduction in hepatic VLDL-TGFA synthesis did not occur in these subjects, there appears to be a significant positive relationship between hepatic de novo lipogenesis and plasma TG concentration in control subjects that was not evident in type 2 diabetes subjects. The 24h measure of TG synthesis may not have captured variability and short-term response during the postprandial period. Further research is required to delineate the contribution of postprandial hepatic TGFA synthesis following diets of specific composition in subjects with type 2 diabetes.

3.6 REFERENCES CITED IN CHAPTER 3

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CHAPTER 4

THE EFFECT OF HIGHER VERSUS LOWER FAT INTAKE ON VLDL TRIGLYCERIDE COMPOSITION IN DIABETIC AND NON-DIABETIC SUBJECTS.

4. 1 INTRODUCTION

Triglyceride (TG) from the liver is secreted as VLDL into the blood as a source of fat for the body during fasting. During feeding, chylomicrons enter the bloodstream and compete with VLDL for clearance, driving a further increase in plasma TG level. Insulin normally counteracts these effects during the fed state by decreasing VLDL synthesis, suppressing plasma free (non-esterified) fatty acid release from adipose tissue and increasing lipoprotein lipase activity. In insulin resistance and diabetes these systems abate, resulting in the fasting and postprandial elevations of plasma TG and free fatty acid that are characteristic of the disease state.

There is some evidence that fatty acids and other substrates are handled differently by the body depending on the amount and type of fat in the diet (McCargar *et al*, 1989). Dietary fatty acids influence the fatty acid composition of adipose tissue (Field *et al*, 1985), incorporation into membranes (Bowen & Clandinin, 2002;Field *et al*, 1990) and lipoproteins (Goh *et al*, 1997;Layne *et al*, 1996). Consequently, these fatty acids influence substrate utilization, oxidation (Clandinin *et al*, 1995;Jones *et al*, 1985), membrane function and cellular processes (Clandinin *et al*, 1993). If dietary composition of fat is important in lipid and lipoprotein metabolism, then it follows that circulating fatty acids likely also play a role. VLDL are the main source of fatty acids for the body during fasting, but are also present and may increase after feeding due to competition for clearance with chylomicrons. Continual lipolysis of these lipoproteins puts the component fatty acids in direct contact with cells of the body. Since the fatty acids released are composed of both exogenous and endogenous sources, examining the relationship between diet and VLDL fatty acid composition may add important information to understanding of alterations in metabolism that occur in diabetes.

Elevated fasting plasma TG indicates an excess of serum TG-rich lipoproteins, including VLDL, chylomicrons and their remnants (Ooi & Ooi, 1998). Hypertriglyceridemia is an independent risk factor for cardiovascular disease (Austin, 1999;Hokanson & Austin, 1996;Sprecher, 1998) and increases risk of major cardiac events independent of HDL or LDL cholesterol concentrations (Assmann *et al*, 1998;Tai *et al*, 1999). However, in Western society today, individuals are in the postprandial state for the majority of each 24h period. Postprandial lipemia seems to have a greater magnitude and duration in patients with type 2 diabetes and has recently been the focus of much investigation due to a relationship to cardiovascular disease risk. Normally, insulin acts to reduce VLDL production and the release of free fatty acids into the bloodstream from adipose tissue. In diabetes these effects seem to be reduced due to resistance of the liver and adipose tissue to the actions of insulin. Furthermore, the combination of elevated plasma free fatty acids, hyperglycemia and chylomicron TG entering the bloodstream following a meal favours VLDL-TG overproduction. As a result, clearance mechanisms may become overwhelmed.

Since nutritional and hormonal changes underlie lipid metabolism, the influence of diet may be important to production and clearance of VLDL-TG. Substrate from the diet has the potential to influence plasma TG level, glycemia and the availability of fat for transport around the body. High carbohydrate intake is known to increase TG levels and this may be even more important in the context of insulin resistance. In general, the more carbohydrate increases and fat intake decreases, the greater the corresponding increase in plasma TG levels. It is thought that carbohydrate induction of hypertriglyceridemia can be prevented by several dietary factors, such as higher fat level (Parks, 2001). Increasing dietary fat intake has long been a controversial therapeutic approach, however the Canadian Diabetes Association recognizes that a higher fat intake (i.e. ≥30% of total daily energy) may be acceptable if it is "primarily composed of mono- and polyunsaturated fats and is low in *trans* fatty acids." Studies have shown that in individuals with type 2 diabetes, polyunsaturated fatty acids have a beneficial effect (Goh *et al*, 1997) and saturated fatty acids have an adverse effect on plasma lipids, whereas monounsaturated fatty acids tend to be neutral or beneficial to plasma insulin, glucose and lipid concentrations (Garg *et al*, 1994;Garg, 1998;Rivellese *et al*, 1994). In general, when compared to higher carbohydrate, higher monounsaturated fat intake results in lower plasma TG concentration, glucose and blood pressure, higher HDL concentrations and (Appel *et al*, 2005;Garg, 1998) and has been associated with a lower risk of cardiovascular disease (Tanasescu *et al*, 2004).

The type of dietary fat also appears to affect postprandial TG levels. It seems that high saturated fat (versus high polyunsaturated fat) intake, particularly over the long term, may impair clearance of triglyceride-rich lipoproteins during the postprandial period (Bergeron & Havel, 1995;Weintraub *et al*, 1988). This research indicates that postprandial VLDL-TG concentration may still be significantly higher than baseline at 6h and takes longer to return to baseline levels than chylomicron-TG (up to 9h). Recently, the focus has turned from dietary fat composition to dietary carbohydrate intake and circulating fatty acid composition (Forsythe *et al*, 2008;King *et al*, 2006;Raatz *et al*, 2001). Like dietary fat quality, serum fatty acid "quality" may be an important long term factor in the development of disease (Warensjo *et al*, 2005).

This purpose of this research was to compare the effect of higher versus lower fat intake on postprandial plasma triglycerides and VLDL-triglycerides in diabetic and matched non-diabetic subjects. Also to test if the fatty acid composition of VLDL triglyceride varies depending on the carbohydrate and fat composition of the diet, and if the response to diet composition differs in diabetes. It is hypothesized that diabetes and/or lower fat intake will result in higher fasting and postprandial plasma triglycerides and that these differences will be reflected in VLDL-triglyceride levels. It is specifically hypothesized that a higher composition of palmitate and stearate and lower composition of oleate will characterize the fatty acid composition differences between diabetes/lower fat intake and non-diabetes/high oleate intake, perhaps due to effects of fatty acid composition on production or clearance of VLDL-TG.

4.2 SUBJECTS AND METHODS

4.2.1 STUDY PARTICIPANTS

Eleven subjects with type [illegible] diabetes and 10 non-diabetic (control) subjects were recruited through the outpatient Metabolic Clinic at University of Alberta Hospitals, Capital Health Authority diabetes registry and a participant database compiled from respondents to another study. All volunteers provided informed consent after all procedures and potential risks were explained. An information sheet was filled out that included a case history and list of food dislikes for menu adjustment. The Faculty of Agriculture, Forestry and Home Economics and the Human Research Ethics board at the University of Alberta approved the study design and procedures.

Baseline screening included weight and height (BMI), waist and hip measurements. All volunteers had 12h fasting blood drawn at the University of Alberta Outpatient Labs for determination of plasma total, LDL and HDL cholesterol, TG, glucose, free fatty acids, insulin, creatinine, C-peptide, ALT, alkaline phosphatase, HbA1c, as well as apoE genotype (Table 1). Volunteers with TG levels >4.0 mmol/L and not in reasonable metabolic control were excluded, as well as those taking drugs that could alter lipid metabolism (i.e. lipid-lowering drugs). Participants on other medications (including Metformin, Levothyroxine and some hypertensive drugs) were instructed to continue and record their normal medication. Test results and medications were reviewed by an endocrinologist.

Table 4-1. Baseline characteristics of subjects completing the study protocol. Diabetic and non-diabetic (control) subjects were matched for gender, age and BMI. Baseline measurements were taken a minimum of one month before beginning study protocol.

	Control Group		*Diabetes Group*
Gender (M/F)	3/4		3/4
Age (yr)	51.4 ± 9.2		50.0 ± 8.8
BMI (kg/m2)	33.5 ± 8.3		33.2 ± 7.5
Weight (kg)	93.4 ± 24.1		91.9 ± 15.4
Waist (cm)	105.6 ± 15.8		105.6 ± 13.5
Alkaline Phos	77.0 ± 26.9		72.0 ± 16.7
ALT	25.0 ± 12.2		29.4 ± 14.0
Creatinine	83.1 ± 18.6		70.3 ± 15.3
Glucose (mmol/L)	5.0 ± 0.4	*	6.2 ± 1.1
HBA1c (%)	5.3 ± 0.4	*	5.9 ± 0.5
Insulin (U/ml)	9.9 ± 7.7		13.6 ± 7.9
C-Peptide (nmol/L)	1.1 ± 0.4		1.5 ± 0.5
HOMA	2.22 ± 1.7		3.83 ± 2.4
FFAs (mmol/L)	0.88 ± 0.4		0.66 ± 0.3
TG (mmol/L)	1.35 ± 0.4		1.96 ± 0.8
TC (mmol/L)	5.41 ± 0.8		4.76 ± 0.6
HDL (mmol/L)	1.34 ± 0.2		1.21 ± 0.2
LDL (mmol/L)	3.46 ± 0.7	*	2.67 ± 0.6
TC/HDL	4.09 ± 0.6		4.01 ± 0.6
ApoE Genotype (E2/E3,E3/E3,E3/E4,E4/E4)	2,3,2,0		3,3,0,1

Values are mean ±SD. * denotes significant difference, p≤0.05.

4.2.2 STUDY DESIGN

Subjects were examined after two feeding periods comprising a total of 3 days each with a one month washout period. To measure macronutrient content of background diet, individuals were instructed to document all food, drink and medications on a daily basis for a period of 7 days immediately preceding the feeding periods, concluding the evening before the first diet day. Subjects arrived at the Human Nutrition Research Unit that evening to pick up packaged meals and were instructed to exclude alcohol intake, not to exercise vigorously, to consume only the food and beverages provided (except calorie-free fluids), and to return containers unwashed as an indication of compliance.

Diets were formulated to meet subjects' energy needs using the Harris Benedict equation and an activity factor. The high carbohydrate/low fat diet was designed to consist of <25% of energy from fat and emphasized complex carbohydrate while limiting simple sugars and high glycemic index foods. The high fat/low carbohydrate diet consisted of the same menu items as the high carbohydrate/low fat diet (Table 4-2) with the isocaloric addition of canola oil to achieve ~35% of energy from fat as mostly monounsaturated fat. Some safflower and flax oil was added to the lower fat diet in order to increase the polyunsaturated fat contributed by the canola oil. Test day breakfast contained the same fat and carbohydrate composition as that of the dietary treatment period providing 1/3 of daily caloric intake (average for all subjects was ~700kcals; Table 4-3). Each meal was similar in energy and macronutrient composition and identical meals were consumed on all 3 diet days. Feeding of subjects allowed control of intake, blinding and randomization of diet treatment order. Each subject completed both treatments, thus serving as their own control.

Table 4-2. Menu items consumed for higher and lower fat diets.

Breakfast	Lunch	Dinner
Orange juice	*Whole wheat rotini	*Turkey sandwich
*Blueberry oatbran muffins	*Tomato base pasta sauce (Vegetables, beef, mozzarella)	Raspberry newtons
*Scrambled egg	*Peas	Melon pieces
*Bread	Apple	

Meals were identical on all 3 days of diet intervention for both higher and lower fat diets.
To meet specific fatty acid amount and composition, safflower oil, flax oil and 50% less fat margarine were added to lower fat items; canola oil and canola margarine were added to higher fat items as indicated by *.

4.2.3 ANALYTICAL METHODS

Composition of background diet and study diet was calculated using Food Processor II nutrient analysis computer software (V9.6.2, Esha Research 2004). Computer determination of study diet composition included integrated fatty acid profiles from Gas-Liquid Chromatography (GLC) analysis of oils and fats (not shown). Test meals were prepared according to calculated recipes and fat was extracted and analyzed by GLC to confirm fatty acid composition of meals (Table 4-4).

Table 4-3. Diet composition of the 2100 kcal total diet and 700 kcal test meal.

	Test Meal Breakfast		Total Diet/day	
Nutrient	**LF**	**HF**	**LF**	**HF**
Protein	15	14	18	16
Carbohydrates	**59**	**49**	**59**	**48**
Dietary Fiber g	12	9	38	31
Fat	**25**	**37**	**23**	**37**
Saturated Fat	5	7	5	6
Monounsaturated Fat	**7**	**20**	**6**	**20**
Polyunsaturated Fat	10	7	10	8
***Trans* Fatty Acid**	<1	<1	<1	<1
Cholesterol mg	273	291	370	388
PUFA:SFA	2.0	1.0	2.0	1.2

Meals of similar fat and fibre composition were designed and adjusted to each subjects' energy requirements. Values are mean % of energy (unless otherwise noted) from a 2100 kcal diet (average intake for all groups) as calculated in Food Processor after GLC of added oils and margarine fat composition was added to the database. LF: lower fat diet, HF: higher fat diet.

Table 4-4. Fatty acid composition of diets fed as analyzed by GLC.

	Test Meal Breakfast		Total Diet/day	
Fatty acid	**LF**	**HF**	**LF**	**HF**
Saturated fatty acids	**6**	**8**	**4**	**6**
14:0 - Myristic acid	0.4	0.6	0.3	0.5
16:0 - Palmitic acid	4	5	3	4
18:0 - Stearic acid	1.2	1.6	0.9	1.3
Monounsaturated fatty acids	**11**	**22**	**8**	**22**
18:1 - Oleic acid	7	18	8	21
Polyunsaturated fatty acids	**10**	**8**	**11**	**9**
18:2 - Linoleic acid	9	7	8	7
18:3 - Linolenic acid	1.5	1.6	2.3	2.0
Other fatty acids	1	1	1	1
PUFA:SFA	1.8	1.1	2.5	1.5

After formulation, meals were prepared and analyzed for fatty acid composition. Values are mean % of energy (except PUFA:SFA) from a 2100 kcal diet (average intake for all groups). LF: lower fat diet, HF: higher fat diet.

An intravenous catheter was inserted into the forearm of each subject for ease of blood collection. A fasting blood sample (20mL) was taken at 07:30h into vacutainer tubes containing disodium EDTA. The test meal breakfast was consumed within 15 minutes of serving and then subjects fasted for the following 8 hours. Blood was collected at 2, 4, 6, and 8h after consumption of the breakfast meal. Lunch meals were served immediately and dinner meals were sent home with subjects to be consumed a minimum of 12h before the final blood draw the next morning at approximately 0900h.

On test day 1 (diet day 3) of each diet treatment, fasting blood samples were obtained following a 12h overnight fast. Subjects abstained from alcohol consumption during the 3 day diet and refrained from strenuous exercise for 24 hours before the test day. Subjects reported to the research centre between 07:15 and 07:30 hours and a flexible venous catheter was inserted into the forearm and blood collected into 3 vacutainers. The first contained lithium heparin for determination of total plasma lipid, glucose and insulin concentrations and was immediately sent to the University of Alberta Hospitals Laboratory for analysis using automated enzymatic procedures. The other 2 tubes contained sodium heparin for all remaining determinations.

Plasma was separated by centrifugation at 1000 x g for 10 minutes at 4°C, immediately extracted and refrigerated. Extraction of VLDL-TG fatty acids involved ultracentrifugation of plasma to separate out chylomicrons within 24 hours of collection. This single centrifugation is sufficient to remove all chylomicrons and chylomicron remnants. Plasma samples were dialyzed and layered under a discontinuous gradient of salt solutions of different densities and subjected to nonequilibrium density gradient ultracentrifugation (Musliner *et al*, 1991). Lipoprotein fractions were sequentially removed and TGs extracted from the VLDL portion as previously described (Layne *et al*, 1996). The procedure involves a Folch extraction, addition of internal standard (17:0), separation of TGs by thin layer chromatography, quantitative recovery of the TG fraction and subsequent methylation (Hargreaves & Clandinin, 1987). Fatty acid methyl esters were dissolved in hexane and 0.5-3.0 μL was injected into a Varian 3600CX Gas Chromatograph. Peaks for fatty acid methyl esters were identified and quantitated using internal standard procedures. These GLC operating conditions are capable of separating methyl esters of all saturated, cismonounsaturated and cis-polyunsaturated fatty acids from 14 to 24 carbons in chain length. For the purposes of this study, only analyses of the major fatty acids (14 to 18 carbons) were included in the results. Amounts of 20-24 carbon fatty acids in the VLDL-TG comprised <4% µg per mL plasma.

4.2.4 STATISTICAL METHODS

Statistical analyses involved the use of GraphPAD Prism (V5.0, GraphPAD Software, San Diego, CA) and SAS software (V8.0, SAS Institute Inc., USA). Paired t-tests were used to compare the effect of lower or higher fat diet within each group. Wilcoxon matched pairs t-tests were used to test differences between diabetes and control groups for each diet. Repeated measures 2-factor ANOVA (fasting measures) or 3-factor ANOVA (postprandial measures) with Bonferroni posttests were used for posthoc comparisons. Sample size calculations based on previous results expecting a decrease in plasma TG concentration of 25% estimated completion of 7 subjects as sufficient statistical power to achieve significance. The level of statistical significance was set at a P-value < 0.05.

4.3 RESULTS

Only subjects complying with and completing both diets were included in the results (n=7 per group; Table 4-1). The major obstacle to recruitment was the exclusion of diabetic respondents who were taking lipid-lowering medications. Of those participants that dropped out, four withdrew at the outset; two after completing one diet period and one participant was ill on the test day and therefore unable to consume the experimental diet.

4.3.1 DIETARY ANALYSES

Most of the subjects included in the results consumed all food as suggested, evidenced by returned food containers. In the case of the other subjects (n=3), the uneaten food from the first diet was weighed and the equivalent caloric amount of the same item was subtracted from the second diet. All items from the second diet were consumed in their entirety. Diets were designed to appear identical and subjects were blinded as to which diet they were consuming first and second. Upon completion of the study, a questionnaire was administered in order to estimate blinding success. Nine out of the 14 subjects were unable to correctly discriminate the diet that was lower or higher in fat, 4 identified both diets correctly and one questionnaire was unreturned.

The week before each diet was fed, 7-day food records were collected and analyzed to determine average energy and macronutrient intake for the subjects' usual diet. Average energy was estimated at 2269 and 2197 kcal for diabetes subjects, 2269 and 1725 kcal for control subjects preceding the higher and lower fat diets, respectively. Average macronutrient intake, expressed as % of energy from protein:carbohydrate:fat was calculated to be 17:48:35 and 17:48:36 for the diabetes group, 17:48:35 and 18:49:34 for the control group preceding the higher and lower fat diets, respectively.

4.3.2 FASTING MEASURES

There were no differences found between diabetes and control groups, or between higher and lower fat intake for any of the fasting measures, with the exception of glucose and VLDL-triglycerides (Figure 1). For fasting plasma glucose following 3 days of lower fat intake, the diabetes group had a significantly higher concentration at 5.8±0.7 mmol/L, whereas the control group had a glucose level of 4.9±0.3 (P=0.04). For fasting VLDL-triglycerides, the control group had a significantly lower concentration after 3 days of higher fat than following lower fat intake (P=0.03). A wide range of concentration was exhibited, with VLDL-triglyceride concentration ranging from 1.2 to 3.9 and 1.2 to 3.8 mmol/L in the diabetes group and 0.8 to 2.0 and 0.6 to 2.7 mmol/L in the control group, following lower and higher fat intake, respectively.

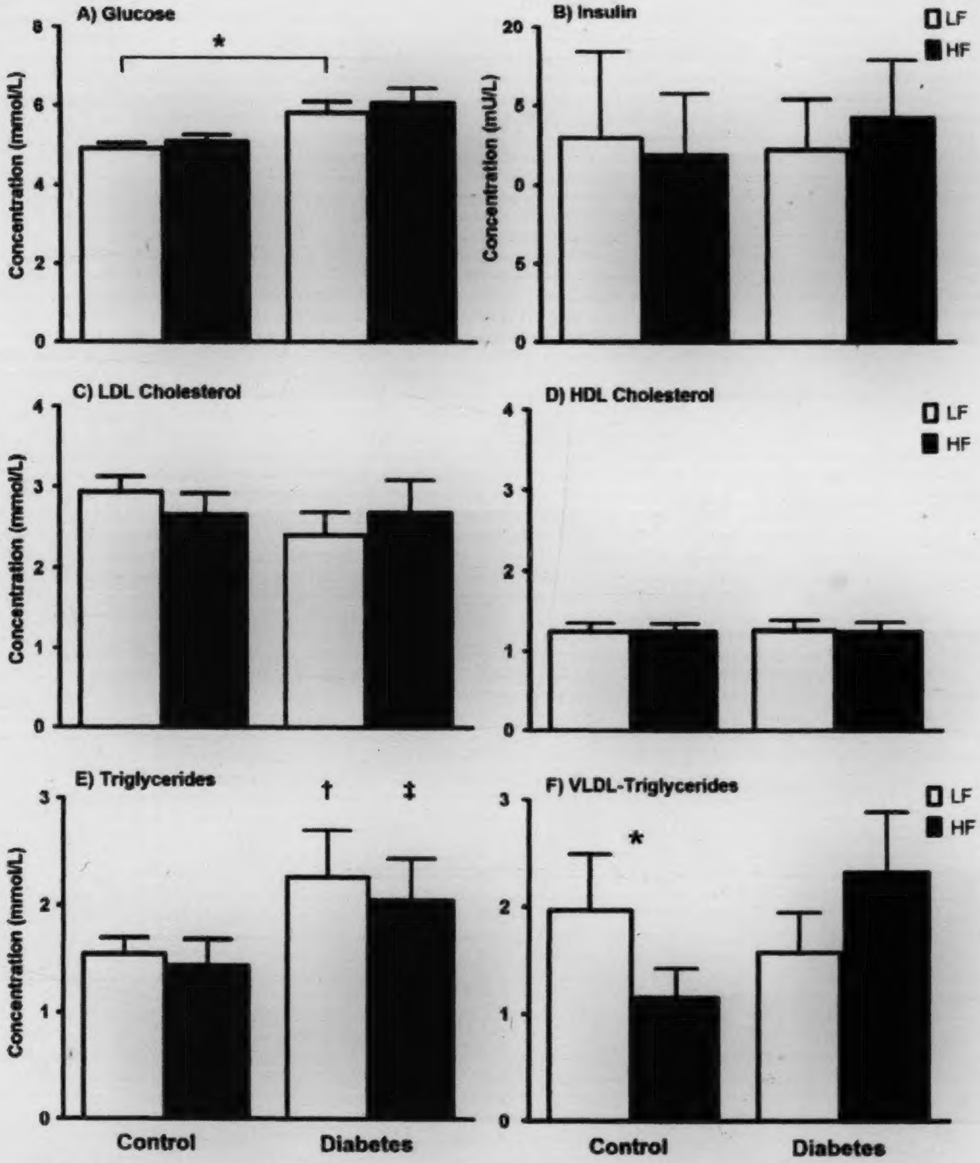

Figure 4-1. Fasting plasma concentrations of clinical and lipid parameters. A) Glucose, B) insulin, C) LDL cholesterol, D) HDL cholesterol E) triglycerides and F) VLDL-triglyceride fatty acid concentrations were measured in control and diabetes subjects following 3 days of lower (LF) and higher (HF) fat intake. Values are means with SEM error bars, * indicates a significant difference at $P<0.05$ as determined by paired t-tests (within groups) or Wilcoxon matched pairs t-tests (between matched subjects). A main effect of diabetes is denoted by † = $P<0.5$ and ‡ = $P<0.01$.

4.3.4 POSTPRANDIAL MEASURES

Postprandial plasma glucose and insulin concentrations did not differ between diets for either the diabetic or control groups. Following the test meal, the 2h postprandial glucose and insulin levels were significantly higher in the diabetes group after consuming the lower fat diet than the control group when consuming either lower or higher fat diets (Figures 1 and 2). The diabetes group had a significantly higher glucose concentration 2 hours after consuming the lower fat diet than the control group at the same timepoint following lower or higher fat intake (P<0.01 and P<0.001)(Figure 2). The lower fat diet also resulted in higher insulin concentrations at 2 hour postprandially in the diabetes group than the control group after consuming either diet (P<0.05)(Figure 3).

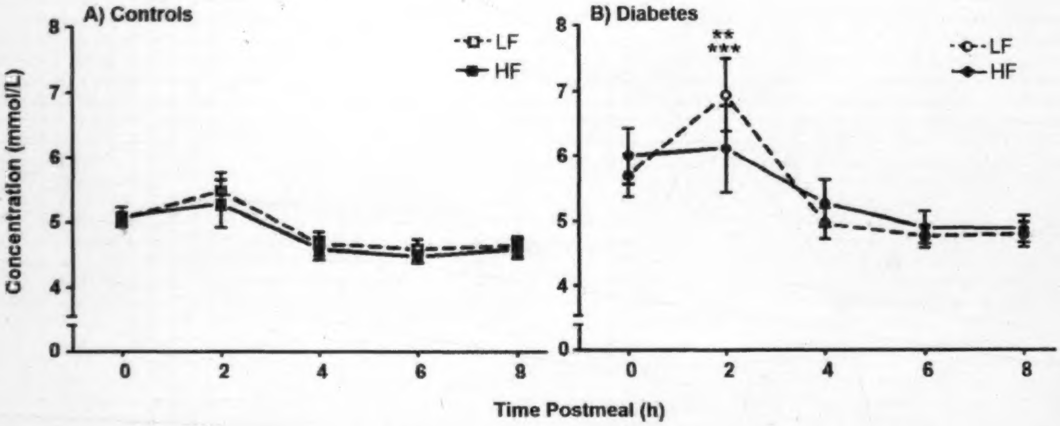

Figure 4-2. Postprandial plasma glucose concentration. A) Control and B) diabetes subjects fasted for 12h (0h), consumed a meal lower (LF) or higher (HF) in fat and postprandial measures were made at 2, 4, 6 and 8 hours. Values are means with SEM error bars, ** and *** indicate a significant difference from control LF and control HF at P<0.01 and P<0.001, respectively.

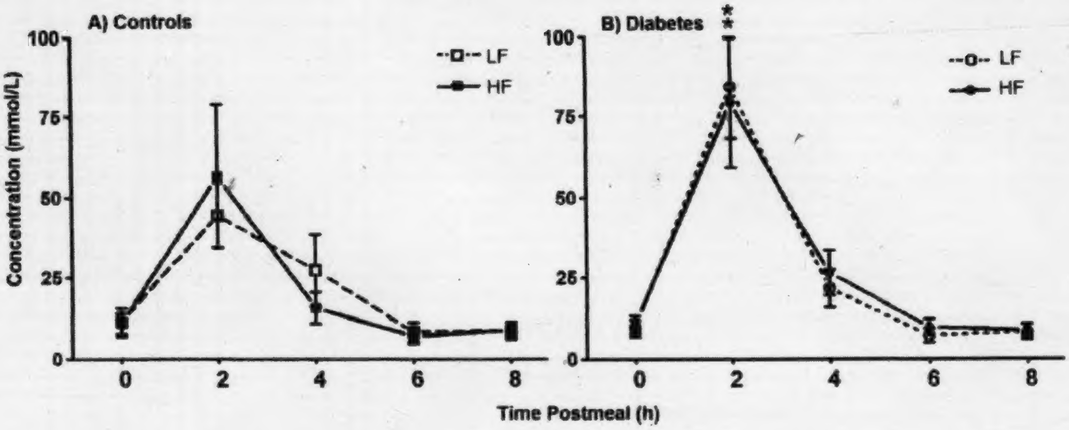

Figure 4-3. Postprandial plasma insulin concentration. A) Control and B) diabetes subjects fasted for 12h (0h), consumed a meal lower (LF) or higher (HF) in fat and postprandial measures were made at 2, 4, 6 and 8 hours. Values are means with SEM error bars, * indicates a significant difference from control LF and control HF at P<0.05.

The relationship between plasma insulin and glucose concentrations was used to estimate insulin sensitivity (HOMA-IR = fasting plasma insulin x fasting plasma glucose/22.5). The diabetes group had a higher 2h HOMA following both diets, indicating that both the higher fat and lower fat diets aggravated glucose disposal shortly after a meal.

In general, triglycerides peaked 4h after the meal, but the diabetes group remained higher at 6h (52%) and 8h (68%), in addition to higher premeal levels (0h; 51%)(Figure 4-4). Overall, there was a main effect of diabetes (LF p<0.002; HF p<0.02) on plasma TG concentration. Postprandial plasma TG concentration increased significantly over fasting levels in both groups following both diets and time had a significant effect on TG levels in the control group when comparing LF and HF diets (p<0.02). The HF diet in the diabetes group resulted in the largest increase in TG (2.39 to 3.64 mmol/L), whereas the LF diet resulted in a trend towards a higher AUC than the HF diet for both diabetes (LF: 20.3, HF: 18.9) and control (LF: 14.2, HF: 13.9) groups. Postprandial LDL and HDL cholesterol levels did not change significantly over time, nor were there any differences between groups or diets.

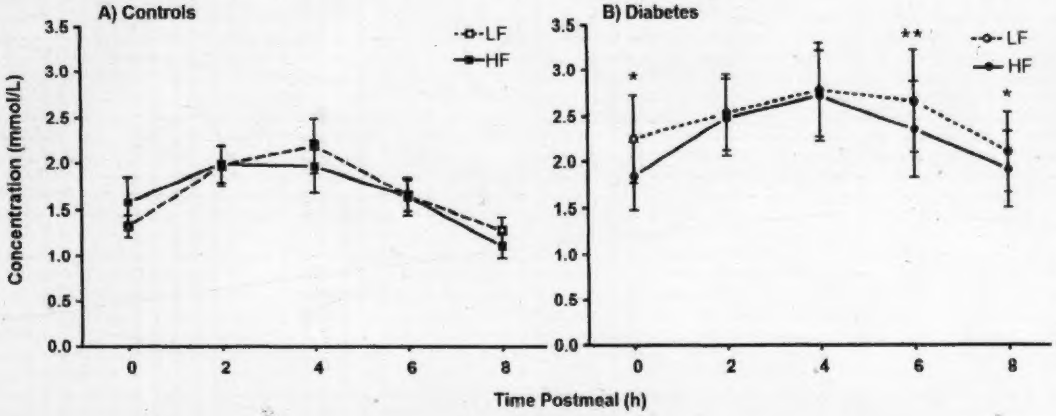

Figure 4-4. Postprandial plasma triglycerides. Fasting (0h) and postprandial (2, 4, 6, 8h) plasma triglyceride concentration following lower (LF) and higher (HF) intake. Values are represented as means, error bars = SEM, * (p<0.05) and ** (p<0.02) indicate timepoints where there was a main effect of diabetes.

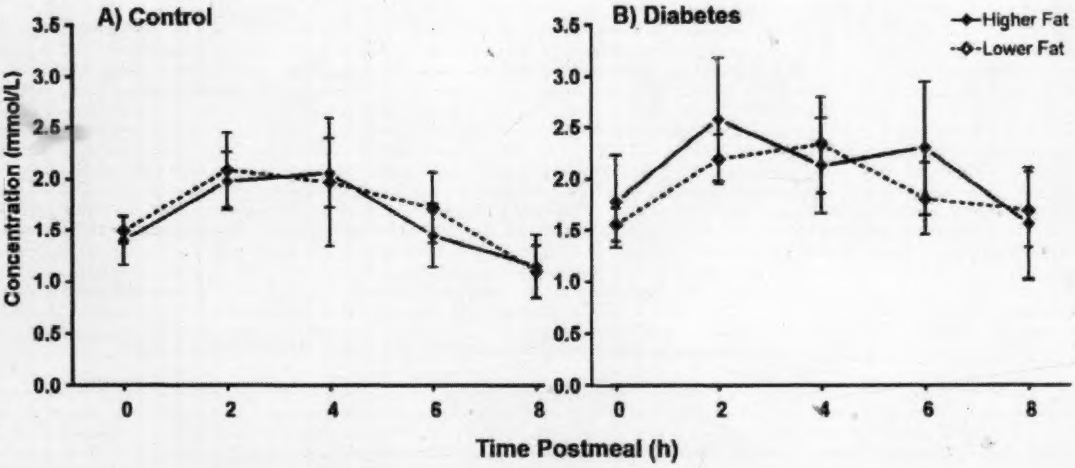

Figure 4-5. Postprandial VLDL-triglycerides. Fasting (0h) and postprandial (2, 4, 6, 8h) VLDL-triglyceride concentration following lower (LF) and higher (HF) intake. Values are represented as means, error bars = SEM, no significant differences were detected.

4.3.5 VLDL-TRIGLYCERIDE FATTY ACID COMPOSITION

It was previously shown (Figure 1F) that fasting VLDL-TG fatty acid level was significantly lower following higher fat intake in control subjects. Figure 5 shows the fatty acid composition of the VLDL-TG. Whereas all fatty acids tended to be higher following higher fat than lower fat intake in the diabetes group, the opposite trend was evident in the control subjects. The fatty acids significantly reduced following higher fat intake in controls were myristate (14:0, P=0.018), palmitate (16:0, P=0.013), palmitoleate (16:1, P=0.018) and linoleate (18:2, P=0.024).

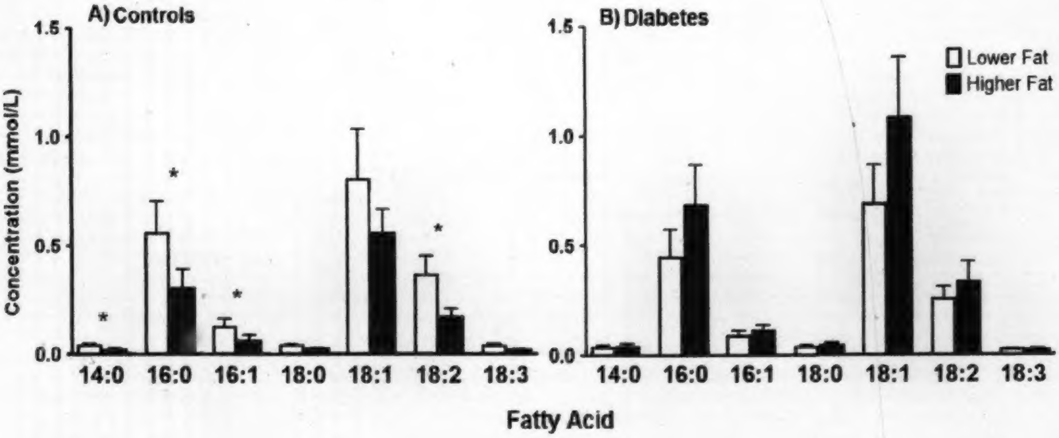

Figure 4-6. Fatty acid composition of fasting plasma VLDL-triglyceride. A) Control and B) diabetes subjects consumed lower or higher fat intake for 3 days. Values represent mean VLDL-TG fatty acid, error bars = SEM and * indicates a significant difference between diets (P<0.05). No differences were found between groups for either diet.

The amount of stearate (18:0), oleate (18:1) and α-linolenate (18:3) in VLDL-TG were also reduced after higher fat intake, but failed to reach significance. Following 3 days of higher fat intake, the mean fasting concentrations for palmitate and 18:1 in diabetes subjects were almost double that in control subjects, but was only a trend (both P=0.078). There were wide ranges of concentration for these fatty acids: 0.1-0.8 and 0.08-1.2 mmol/L for 16:0 and 0.2-1.1 and 0.2-2.1 mmol/L for 18:1 in control and diabetes subjects, respectively.

Analysis by repeated measures ANOVA did not reveal any significant differences between groups or diets for individual fatty acids in postprandial VLDL-TG (Figure 4-6 and 4-7).

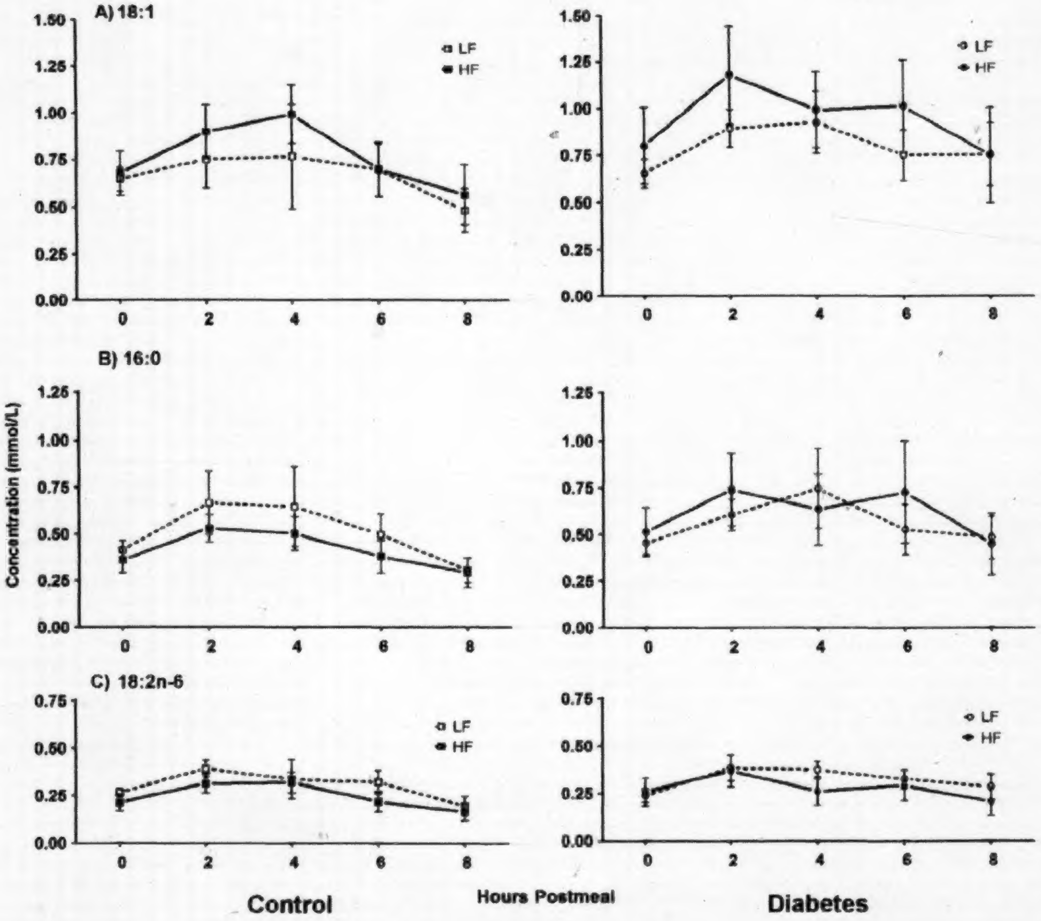

Figure 4-7. VLDL-triglyceride postprandial composition: major fatty acids. The plasma concentration of major fatty acids in the VLDL triglyceride of control (left panel) and diabetes subjects (right panel) following lower fat (LF) or higher fat (HF) intake are shown: A) oleic acid (18:1), B) palmitic acid (16:0) and C) linoleic acid (18:2). Values represent mean, error bars = SEM. No significant differences were detected by repeated measures ANOVA within subjects or between groups.

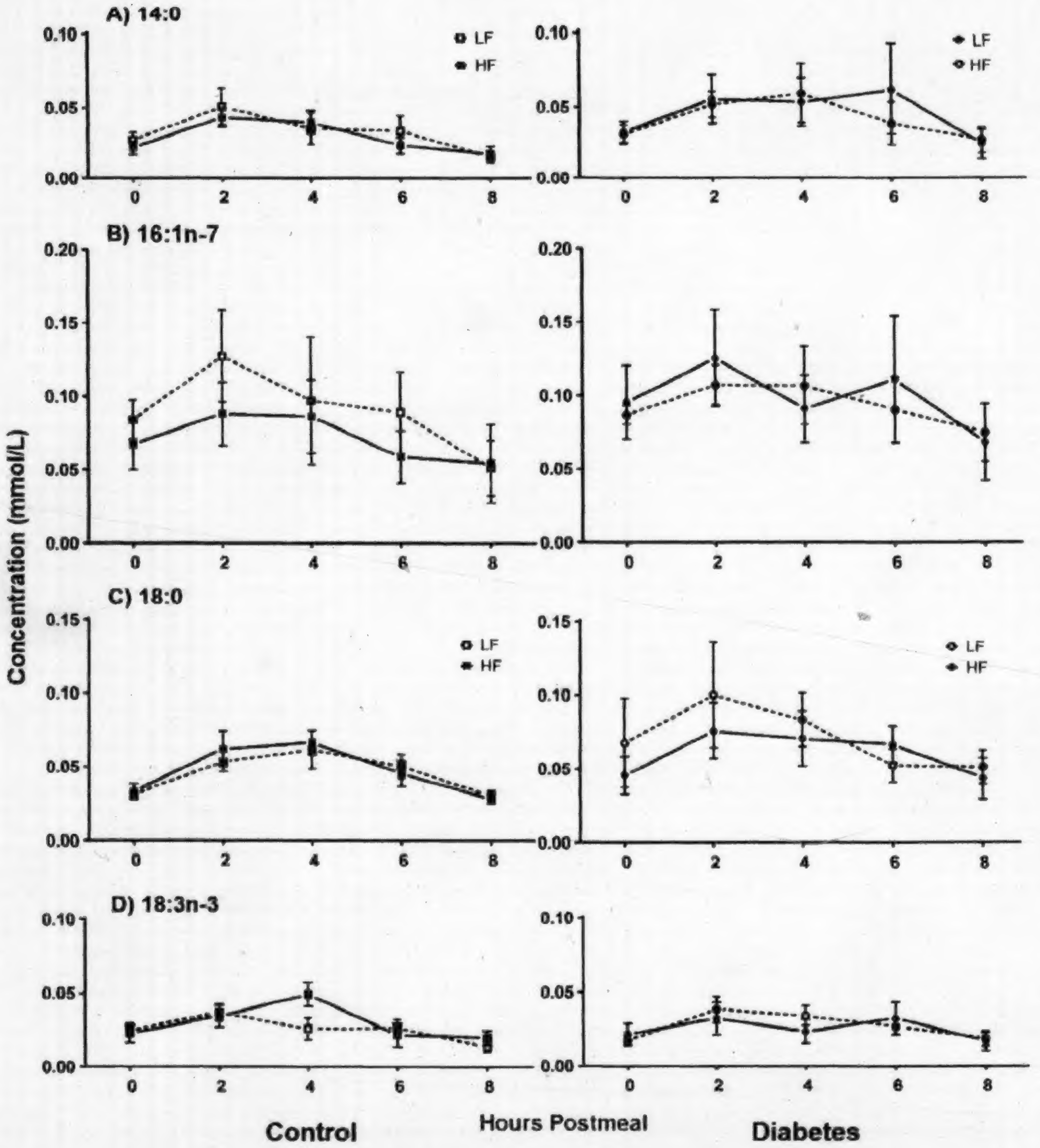

Figure 4-8. VLDL-triglyceride postprandial composition: minor fatty acids. The plasma concentration of minor fatty acids in the VLDL triglyceride of control (left panel) and diabetes subjects (right panel) following lower fat (LF) or higher fat (HF) intake are shown: A) myristic acid (14:0), B) palmitoleic acid (16:1), C) stearic acid (18:0) and D) α-linolenic acid (18:3n-3). Values represent mean, error bars = SEM. No significant differences were detected by repeated measures ANOVA within subjects or between groups.

Relationships between VLDL-TG fatty acids and clinical measures

There were no correlations found between fasting plasma TG concentration and VLDL-TG fatty acid concentration except for VLDL-TG stearic acid concentration in diabetes subjects when consuming the lower fat diet (R^2=0.82, P=0.02). The strongest relationship existed between insulin resistance (as indicated by HOMA-IR) and fasting VLDL-TG myristic acid (14:0) concentration. These two factors were related for both lower fat (R^2=0.53, P=0.05) and higher fat (R^2=0.70, P=0.005) diets (Figure 4-8). When the groups were separated into control and diabetes, this relationship remained for control subjects after lower fat intake (R^2=0.82, P=0.023) and diabetes subjects after higher fat intake (R^2=0.98, P<0.0001).

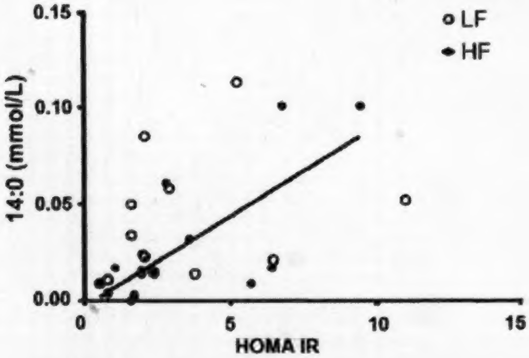

Figure 4-9. The relationship between HOMA-IR and myristic acid (14:0) in VLDL-TG. There was a significant relationship between HOMA-IR and 14:0 level after 3 days of higher fat (HF; R^2=0.70, P=0.005) and lower fat (LF; R^2=0.53, P=0.05) intake in all subjects. — indicates the linear relationship between higher fat HOMA-IR and VLDL-TG 14:0 concentration after higher fat intake.

4.4 DISCUSSION

Reducing fat intake has long been the main focus of nutrition research surrounding diabetes and cardiovascular disease. However, when the type of dietary fat is considered, it is often found to be more important than the total amount of fat (Hu *et al*, 2001). Further, much attention has been given to the influence of fatty acids in dietary triglycerides on atherogenesis and yet research is scant surrounding the fatty acid composition and effects of circulating triglycerides. It is often simply assumed that higher dietary fat leads to higher levels of the same fatty acids in the blood, and therefore higher exposure to cells of the body. Indeed, the triglycerides in chylomicron-TG tend to reflect dietary composition. However, VLDL-TG composition seems to vary from that of diet (Heath *et al*, 2003) and little is known about the influence of dietary fat composition or carbohydrate intake. VLDL-TG transports the majority of serum fatty acids and thus the contribution of saturated fatty acids may be especially important. Results from this research show that when subjects consume the same diet, VLDL-TG fatty acid levels and the types of fatty acids can vary considerably.

It would appear from this research that in non-diabetic control subjects the small reduction in TG concentration after 3 days of higher fat intake is consistent with a significant reduction in VLDL-triglyceride. When VLDL was examined as to which fatty acids contributed to this difference, all of the fatty acids that were measured had decreased, some in significant amounts. That is, myristic, palmitic and palmitoleic acids showed significant reductions, even though levels of these fatty acids were held constant between diets. Linoleic acid was also lower after higher fat intake, and this result is different than that seen for lower fat/higher carbohydrate diets examined in other studies. Previous research involving extreme carbohydrate overfeeding (Aarsland *et al*, 1996) or very low carbohydrate diets (Forsythe *et al*, 2008) have shown that high carbohydrate intake results in higher levels of palmitate and lower linoleic acid in serum lipids, often in disproportion to that provided by the diet. More moderate changes in total dietary fat intake (20% vs. 45% of energy from fat) also seem to result in changes in fatty acid composition of serum TG, as well as phospholipids, cholesteryl esters and free fatty acids. The main effects of lower fat intake seem to be an increase in the proportion of saturated and n-3 fatty acids and decrease in linoleic acid in serum lipids (Raatz *et al*, 2001). It should be noted that the diets fed in these studies were either longer term and/or involved more extreme in macronutrient manipulation than the current study. Also, linoleic acid levels in the diets of this study were equivalent, if not higher in the lower fat diet (Table 4-4), perhaps compensating for any reductions that may have been expected to occur.

It has previously been shown that specific fatty acid composition of VLDL-TG may be regulated through systems of preferential uptake, storage, utilization and/or oxidation and these systems are altered in insulin resistant states. The experimental diet was designed so that the amounts of individual fatty acids remained constant while the difference in fat intake was primarily comprised of oleate. The fact that VLDL-TG oleate level was not significantly changed following higher fat intake was surprising, even though the amount of this fatty acid was at least double that of the lower fat diet. The diabetes subjects in the current study seemed to have differences from the control group in regards to VLDL-TG total fatty acid concentration and individual fatty acids following the same change in diet. A high amount of individual variation in this data resulted in a lack of statistical significance; however all fatty acids were increased to some extent in fasting VLDL-TG. Conversely, there was a significant effect of diabetes on total postprandial TG, and this was demonstrated by higher curves following both higher and lower fat intake, particularly at 6 and 8 hours. This agrees with other research indicating that postprandial lipemia seems to be of a greater magnitude and of longer duration in diabetes and insulin resistance. This study differs from most research examining postprandial lipemia because the test meal fed was not a fat load or "challenge". Traditionally, postprandial fat metabolism has been studied using a fat bolus to study lipid tolerance and the ability of the body to clear TG-rich lipoproteins. Instead, the present study tested the effect of a mixed diet within the usual range of fat and carbohydrate intake, and it seems that in diabetes there remains a 'lipemia' of greater magnitude and duration than in non-diabetic subjects.

Both insulin and glucose are known to affect lipid metabolism through long and short term regulation of enzymes and gene expression. Resistance to insulin action may interfere in many ways, influencing processes from de novo lipogenesis and desaturation/elongation to VLDL synthesis and secretion. It is not surprising that higher carbohydrate intake results in higher plasma glucose levels in diabetes when these diets are high in sugar or glycemic index and low in fibre. However, the diets fed in this study were low glycemic index and high in fibre. The test meals were approximately equal in total sugar, at 50g versus 55g total sugars in the higher and lower fat meals, respectively (data not shown), and yet 2h levels of plasma glucose were higher in the diabetes subjects following the lower fat/higher carbohydrate diet. In order to tease out the the balance between insulin secretion and hepatic glucose output (Wallace *et al*, 2004) or in this case (i.e. postprandially) glucose response to diet, HOMA-IR was also calculated in these subjects as an estimate of postprandial insulin sensitivity. A higher HOMA, as is the case of the 2h value for the diabetes group following both diets (data not shown), indicated that both glucose and insulin are higher in diabetic subjects following a meal. This occured despite fat and carbohydrate intake differences between diets and is likely to have affected fat metabolism of these individuals. Interestingly, the strongest relationships between HOMA and any measure of fatty acid metabolism was with the VLDL-TG saturated and monounsaturated fatty acids, but only during higher fat intake. Myristic acid showed a significant positive relationship with HOMA-IR when subjects consumed either diet, but this correlation was particularly significant in the diabetes subjects after higher fat intake.

Postprandial levels of individual fatty acids were highly variable, however some patterns seem evident. The diabetes group generally seemed to have higher 14:0 in VLDL-TG, whereas the control group appeared to consistently increase 16 carbon fatty acids when consuming the lower fat diet. It is possible that these were released from adipose tissue as plasma free fatty acids, taken up by the liver and packaged as VLDL. The other possibility is that de novo lipogenesis may have been contributing to these pools as these fatty acids are known to be synthesized by humans, and specifically conversion to 16 carbon fatty acids from 14:0 in controls consuming lower fat. Previous studies showing an increase in palmitic acid and a decrease in linoleic acid following low fat/high carbohydrate intake attributed it, in part, to de novo lipogenesis of palmitate from carbohydrate. This was sometimes inferred (Forsythe *et al*, 2008;Raatz *et al*, 2001) or measured more directly using stable isotopes (Hudgins, 2000). Although in general these studies altered the total amount of carbohydrate and fat, it should be noted that there was considerable variance in length and control of diet, hypo- versus hyper- versus -eucaloric, the amount and type of fat and carbohydrate, to name a few. If VLDL-TG palmitoleic levels can be used as an indicator of de novo lipogenesis (Forsythe *et al*, 2008), then the higher fat diet may be suppressing lipogenesis in the control subjects, while not having any significant effect in diabetes subjects (Figure 4-5). If lipogenesis is higher in both control subjects consuming lower fat (upregulation) and diabetes subjects consuming higher fat (absence of downregulation), then perhaps the relationship to HOMA-IR should have been stronger with palmitoleic acid than it was for myristic acid. Although there was a correlation observed between HOMA and palmitoleic acid in VLDL-TG following higher fat intake (data not shown), this relationship was only found in control subjects consuming lower fat, but not in diabetes subjects consuming higher fat.

The difference between fasting fatty acid composition and postprandial fatty acid composition of the VLDL-TG is worthy of examination. There may be a greater contribution of dietary fatty acids to VLDL-TG (via chylomicron or free fatty acid uptake by the liver) in diabetes subjects as indicated by higher total plasma triglycerides, particularly at 6 and 8 hours postprandially. Free fatty acids from the plasma are thought to contribute to the majority of TG fatty acid in the VLDL, but the amount may vary depending on feeding state. During fasting it is likely that free fatty acids come from adipose tissue fatty acid release, whereas following a meal there may be some contribution from dietary TG via spillover from lipolysis of chylomicron TG (Barrows *et al*, 2005). Therefore it is possible that during the fed state, fatty acid composition of VLDL-TG may more closely reflect the recent composition of dietary TG (test diet), whereas during the fasting state it may reflect the composition of long term exogenous source TG (habitual diet) or long term endogenous source TG (de novo lipogenesis).

If postprandial VLDL-TG fatty acids were contaminated with chylomicron remnant TG, this may have influenced analyses of composition. The amount of contamination is unlikely to have much of an influence as chylomicron-TG concentration in plasma tends to be much less than that in VLDL. The majority of plasma TG at any point in time is carried in VLDL, particularly during fasting when chylomicrons are mostly absent from plasma (Schaefer *et al*, 1978). In normolipidemic individuals fed a fat-rich mixed meal, ApoB-48 was undetectable in plasma 9 hours later (Schneeman *et al*, 1993). Previous to 9 hours, since there may be chylomicron particles present and VLDL and chylomicron remnants have similar densities, it is not known whether complete separation by conventional ultracentrifugation is consistent. This is the case even following a meal whereby the contribution of chylomicron-TG to total TG may only reach as high as 20% (Schneeman *et al*, 1993). Even after a large fat load, the greatest increase in postprandial TG may occur in chylomicrons, but the majority of TG is carried in VLDL. Nevertheless, the amount seems to vary greatly between individuals (Cohn *et al*, 1993) and may have been a contributing factor during the postprandial stage and may have accounted for the differences seen in VLDL-TG fatty acid composition in fasting versus postprandial blood.

The short duration of the diet could be viewed as a limitation of this study. Although the applicability to longer term diets is limited, it should be noted that the diet was long enough to account for immediate changes in hormones and gene expression (which occur as early as immediately after meal consumption up to 2h afterwards). The diet fed was also long enough to exert influence on any delayed secretion of fat from either the intestine or from liver TG stores. It has previously been indicated that the meal as early as the night before can affect postprandial lipemia the following morning. Also, estimation of each subjects' previous diet using analyses of 7-day food records showed that habitual macronutrient intake did not differ between diet periods.

Another limitation of the study is the small sample (n=7 in each group). Although power calculations were used determine the sample size required to detect changes in TG concentration, the greater variation in VLDL-TG concentration and fatty acid composition may have required a larger number of subjects in order to detect significant changes. A crossover design was used to increase power and eliminate interindividual differences in response to the diet interventions.

4.5 CONCLUSIONS

Diabetes involves distinct alterations of fat and carbohydrate metabolism, which affect the transport and utilization of exogenous and endogenous fatty acids. In the present study, a short term isocaloric exchange of dietary carbohydrate and fat resulted in significant differences in VLDL-TG fatty acid concentration and fatty acid composition of non-diabetic subjects that were not evident in subjects with diabetes. A decrease in all VLDL-TG fatty acids, particularly saturated fatty acids, occurred in non-diabetic subjects after higher fat intake despite the fact that this diet was >35% of energy from fat, whereas the lower fat diet was <25%. If serum fatty acid profile is associated with development of metabolic perturbations such as insulin resistance, then the effect of carbohydrate intake as well as dietary fat composition may be important in ameliorating disease risk.

There seemed to be a unique effect of diabetes on fatty acid metabolism in this study. Further analyses from this research will delineate whether differences in VLDL-TG composition may be the result of varying input from the sources of fatty acid for VLDL-TG synthesis, such as plasma free fatty acids or hepatic *de novo* lipogenesis. From this research, it appears that differences in VLDL-TG fatty acid composition may depend on both dietary fat level and the presence of insulin resistance.

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CHAPTER 5

THE EFFECT OF SHORT TERM HIGHER VERSUS LOWER FAT INTAKE ON POSTPRANDIAL FATTY ACID COMPOSITION IN DIABETIC AND NON-DIABETIC SUBJECTS:

THE CONTRIBUTION OF DE NOVO LIPOGENESIS.

5.1 INTRODUCTION

Low fat diets are often prescribed to reduce cardiovascular disease risk, but these diets commonly increase fasting plasma triglyceride (TG) levels. Hypertriglyceridemia is the most common dyslipidemia in diabetes and is considered an independent risk factor for cardiovascular disease (Austin, 1999;Hokanson & Austin, 1996;Sprecher, 1998). Elevated plasma TG results from an excess of TG-rich lipoproteins, which includes VLDL, chylomicrons and their remnants (Ooi & Ooi, 1998). A major driver for VLDL synthesis and secretion is the availability of lipid, the sources of which are de novo lipogenesis, chylomicron remnant TG and plasma free fatty acids. It is thought that fatty acids may be sequestered into cytosolic TG stores before incorporation of into VLDL-TG, resulting in a specific fatty acid profile that differs from dietary composition. It is known that de novo lipogenesis contributes to VLDL-TG, but the exact role is unclear. The amount of synthesis varies considerably between individuals, however a consistent relationship has been found between fasting plasma TG level and hepatic de novo fatty acid synthesis (Hudgins, 2000;Konrad *et al*, 1998). This relationship seems to be strongest when subjects consume lower fat/higher carbohydrate diets (Chapter 3, Figure 3-4). Although previous studies have examined the total amount of fatty acids synthesized in vivo, very few have determined which fatty acids are being synthesized, and if this varies between individuals, diets and in metabolic states such as diabetes.

Research has indicated that diets consisting of <30% of energy from fat result in minimal lipogenesis, while high carbohydrate diets, particularly when high in simple sugars, result in significant stimulation of fatty acid synthesis (Hudgins, 2000). It has also been shown that the major product of de novo lipogenesis is palmitate and increased lipogenesis as a result of higher carbohydrate intake results in an increased palmitate and decreased linoleate in VLDL-TG (Hudgins, 2000). Since then, more research has focused on plasma fatty acid composition as a result of dietary fat composition and as a result of dietary fat reduction. Changes observed include an increase in total and saturated fat content in the VLDL fraction following higher carbohydrate intake, as well as in phospholipids and cholesterol esters. This compositional change is often attributed to de novo synthesis of fat from carbohydrate. Sometimes this is assumed because it is known that the major product of human lipogenesis is palmitate. Other research uses markers of lipogenesis such as 16-1n-7 concentration (Forsythe *et al*, 2008) or linoleate dilution (Hudgins *et al*, 2000). In some cases stable isotopes are used to estimate fatty acid synthesis more precisely (Hudgins *et al*, 2000). Very few studies have actually measured de novo lipogenesis of individual fatty acids directly in humans and no studies to date have examined hepatic synthesis of individual fatty acids using deuterium incorporation.

Lipogenesis varies considerably between individuals and is dependent on dietary factors, however, the exact role in modulating fatty acid composition is not known. The contribution of lipogenesis to postprandial lipemia, which seems to have a relationship with insulin resistance, has only recently been considered (Barrows & Parks, 2006). Examining the relationship between diet, plasma TG levels and fatty acid synthesis during fasted and fed states, as well fatty acid composition, may add important information to understanding of the alterations in lipid metabolism that occur in diabetes.

The purpose of this research is to examine how dietary carbohydrate and fat composition may contribute to the atherogenic hypertriglyceridemia that occurs in diabetes. The objectives were to alter the carbohydrate and fat intake in subjects with diabetes versus matched non-diabetic subjects and examine the effect on rate of hepatic de novo lipogenesis, of individual fatty acids synthesized by the liver and the contribution to VLDL-TG fatty acid composition, as well as the relationship to plasma TG levels. Specifically, the objective was to determine if *de novo* lipogenesis contributes to VLDL-TG fatty acid composition, and if the fatty acids synthesized by the liver (i.e. saturated vs. unsaturated) vary depending on the carbohydrate and fat content of the diet and the presence of diabetes. It is hypothesized that de novo synthesis of total and saturated fatty acids is higher in diabetic subjects and following lower fat/higher carbohydrate intake, resulting in differences in VLDL-triglyceride fatty acid composition.

5.2 SUBJECTS AND METHODS

5.2.1 STUDY PARTICIPANTS

As described in Chapter 4.2.1, 11 subjects with type 2 diabetes and 10 matched non-diabetic (control) subjects were recruited and screened. Diabetes and non-diabetic control groups were similar in age, BMI, waist circumference and fasting measurements of plasma total, HDL cholesterol, TG, free fatty acids, insulin, C-peptide, HOMA, creatinine, ALT and alkaline phosphatase. However the diabetes group had significantly higher plasma glucose level (6.2±1.1 vs 5.0±0.4 mmol/L) and HBA1c (5.9±0.5 vs 5.3±0.4%), and the control group had a higher LDL cholesterol level (2.67±0.6 vs 3.46±0.7 mmol/L) (Table 4-1).

5.2.2 STUDY DESIGN

All subjects were fed two diets for 3 days in a blinded randomized crossover design separated by a minimum one-month washout period (Chapter 4.2.2). Subjects were tested at the same time each month to account for hormonal variation in the menstrual cycle. Diets consisted of identical items differing in energy from fat (lower fat <25%; higher fat >35% achieved by addition of canola oil)(Table 4-2).

On test day 1 (diet day 3), a 12h fasting (0h) blood sample was drawn as "background" deuterium enrichment. Subsequently, subjects drank a priming dose of ^2H2O at 1.0g/kg estimated body water (60% of body weight). Immediately following, the test day 'breakfast' meal was consumed containing the same fat and carbohydrate composition as that of the dietary treatment period providing 1/3 of daily caloric intake (average for all subjects was ~700 kcals; Table 4-3). At regular intervals throughout the next 24h, subjects drank a maintenance dose of 1.0 g ^2H_2O/kg estimated body water diluted in 1.5L regular bottled water to maintain plasma levels of deuterium at plateau. Postprandial blood was drawn 2, 4, 6 and 8 hours after test meal consumption. The 'lunch' meal was served immediately following the 8 hour fast and a 'dinner' meal was sent home with subjects to be consumed a minimum of 12h before the next blood draw the following morning. The final 12h fasting blood sample drawn on test day 2 at approximately 09:00h and was used for determination of 24h fatty acid synthesis.

5.2.3 ANALYTICAL METHODS

Diet Composition

Composition of background and study diet was calculated using Food Processor II nutrient analysis computer software incorporating GLC determination of study oil fatty acid composition. Diet achieved a mean lower fat intake of 23% and a higher fat intake of 36% of energy from fat (Table 4-3). Test meals were prepared according to calculated recipes and fat was extracted and analyzed to confirm that fatty acid composition of meals met the diet design for fatty acid composition (Table 4-4).

On test day 1 (diet day 3) of each diet treatment, a fasting blood sample was drawn, the test meal breakfast was consumed and subjects fasted for 8 hours as outlined in Chapter 4.2.3. Blood was collected at 2, 4, 6, and 8h, the remaining meals of the diet were consumed a minimum of 12h before the final blood draw on test day 2. VLDL-TG fatty acids were extracted from plasma samples and fatty acid methyl esters were analyzed by GLC as previously described (Chapter 4.2.4).

Relative and Net Amount of De novo Total Fatty Acid in VLDL-triglyceride

Isotopic analysis of VLDL-triglyceride deuterium (^2H) enrichment was performed by ^2H incorporation techniques using isotope ratio mass spectrometry. Previous research using ^2H incorporation to measure hepatic fatty acid synthesis by Konrad et al. utilized a theoretical equation based on the maximum possible enrichment of VLDL-triglyceride. Briefly, sample enrichment was measured as the relative difference in ^2H abundance from standard mean ocean water (SMOW). The difference is referred to as delta (δ) and expressed in parts per thousand (‰). The maximum possible enrichment of VLDL-TG was considered to be equal to the enrichment of plasma after ${}^2H_2O$ administration, corrected for the maximum number of ${}^2H$ atoms which could enter the newly synthesized fatty acids (Konrad, 1999). The maximum ${}^2H$ incorporation was calculated at 0.87 g-atoms ${}^3H$ per g-atom C based on adipose fatty acid synthesis (Jungas, 1968). It is assumed that a constant fraction of deuterium atoms is incorporated into precursor fatty acids under different metabolic conditions. Thus, a correction factor for plasma deuterium enrichment can be calculated using the ratio of carbon to hydrogen for the average triglyceride molecule and correcting for glycerol carbon atoms (Leitch & Jones, 1991). It was previously observed that the average triglyceride molecule in the VLDL fraction had three monounsaturated fatty acids with 17 carbons (Layne *et al*, 1996) and calculations using data from the present study confirmed this observation. Thus, the correction factor was 0.477 based in the average triglyceride, outlined as follows:

$$\frac{0.87\,{}^2H}{C_{fa}} \times \frac{51C_{fa}}{54C_{glycerol+fa}} \times \frac{54C_{glycerol+fa}}{93H_{fa}} = \mathbf{0.477}\,\frac{{}^2H}{H_{fa}} \qquad (1)$$

As described by Konrad et al. (1998), the ratio between the actual enrichment of VLDL-TG over 24h and the maximum enrichment possible can be used to represent the relative amount of de novo synthesized fatty acid in the VLDL-TG pool. In terms of percent, this relationship can be described in the following equation:

$$\text{DNFAr [\%]} = \frac{\delta VLDLTG_{ENR} - \delta VLDLTG_{BG}}{(\delta PW_{ENR} - \delta PW_{BG}) \times \mathbf{0.477}} \times 100 \qquad (2)$$

Where ENR represents 'enriched' sample or ${}^2H$ enrichment after ${}^2H2O$ administration and BG represents 'background' sample or natural abundance of ${}^2H$ before administration of ${}^2H_2O$ in the VLDL-triglyceride (VLDLTG) fraction and as measured in the plasma water (PW) portion of the sample for each subject. DNFAr is equal to the fatty acid fractional synthetic rate x 100 and may be understood as the % of de novo fatty acid in the entire fatty acid pool measured.

For each subject, an estimate was made of the net amount of de novo synthesized fatty acid in the VLDL-TG pool (DNFAn). This can be calculated from fractional synthetic rate and an estimation of total VLDL-TG pool size as follows:

$$\text{DNFAn [mg]} = (\text{FSR})(\text{VLDLTG [mg/L]})(\text{plasma vol est[L]}) \tag{3}$$

Where FSR is the fractional synthetic rate (DNFAr not expressed as a %) and VLDLTG pool size is estimated from VLDL-TG concentration as measured by quantitative GLC analysis and an estimation of total plasma volume. Plasma volume was estimated for each subject at 37.5 ml/kg for females and 45 ml/kg for males (Dagher *et al.*, 1965).

Relative and Net Synthesis of Individual Fatty Acids in VLDL-triglyceride

For the synthesis of individual fatty acids, the above calculations were used with the correction factor adjusted according to the triglyceride representing each fatty acid rather than the average triglyceride. For example, when the fatty acid in question was palmitate (16:0), the molecule used in the calculation is tripalmitate and the correction factor was determined as follows:

$$\frac{0.87D}{C_{fa}} \times \frac{48C_{fa}}{51C_{glycerol+fa}} \times \frac{51C_{glycerol+fa}}{87H_{fa}} = \mathbf{0.449}\,\frac{D}{H_{fa}} \tag{4}$$

The correction factor for each fatty acid was calculated as: myristic acid 0.487, palmitic acid 0.449, palmitoleic acid 0.480, stearic acid 0.477, oleic acid 0.447, linoleic acid 0.505, and α-linoleic acid 0.540.

It should be noted that relative synthesis rate of individual fatty acids is referred to as DNFAr, which is equal to the fatty acid fractional synthetic rate x 100. This may be understood as the % of each individual fatty acid synthesized de novo in the pool of that individual fatty acid as measured. In other words, DNFAr describes the amount of de novo fatty acid within the entire pool of a fatty acid (deuterium labeled fatty acid + non-deuterium labeled fatty acid).

All samples were run in duplicate on a Gas Chromatograph Pyrolysis Isotope Ratio Mass Spectrometer (GC/P/IRMS; ThermoFinnigan Delta PlusXL; Breman, Germany), which permits qualitative and quantitative determination of specific FAs as well as the atom % enrichment of deuterium (^2H) present in fatty acid methyl esters. Effluent from the gas chromatograph is pyrolyzed in a furnace at 1450°C to form hydrogen or H^2H, carbon monoxide and water. CO and H_2O are removed and the H_2/H^2H mixture passes into the mass spectrometer where the enrichment of ^2H in each peak from the gas chromatograph is measured. The sensitivity of the instrument is 10 nmole on column with an internal precision of 0.25/mil. If data from duplicate samples were not consistent, the sample was reprepared and rerun in duplicate. If the area under the peak for a particular fatty acid was <0.1, the concentration of the fatty acid for the purposes of ^2H enrichment calculations was considered to be negligible. When the instrument separated peaks for isomers the 18:1n-7 and 18:1n-9, peaks were manually selected to include both peaks together and considered as total of the 18:1 isomers.

Plasma Water Deuterium Enrichment

Some important assumptions are made when using the ^2H incorporation method. It is assumed that cell membranes are permeable to $^2H_20$ and that plasma deuterium enrichment is identical to that of the intracellular pool from which the VLDL-TG incorporates ^2H.

Isotopic analysis of plasma water deuterium enrichment was performed using standard vacuum techniques (Jones *et al*, 1988). Pyrex tubes containing 60mg zinc were evacuated and flushed with nitrogen gas. A 2µl capillary tube filled with plasma was added to the tubes, which subsequently immersed in liquid nitrogen, frozen, evacuated and sealed. Samples were reduced to hydrogen at 510°C for 30 min under 10^{-3} torr (where 1 torr = 1.333.322 $N \cdot m^{-2}$). Sample ^2H enrichment was analyzed using a 903D dual-inlet isotope ratio mass spectrometer (IRMS; VG Isogas, Cheshire, UK). The mass spectrometer was calibrated by using Vienna standard mean ocean water, 302B, and Greenland Ice Sheet Precipitation standards obtained from the International Atomic Energy Agency. The dilution space of each subject was obtained from plasma ^2H enrichments using the equation:

$$N = [WA(\delta_a - \delta_t)]/[18.02a(\delta_s - \delta_p)] \qquad (5)$$

Where N (mol) is the dilution space, W (g) is the amount of tap water used to dilute the dose for analysis, A (g) is the amount of mixed dose given to the subject, a (g) is the dose diluted for analysis, and δ (‰) is the enrichment of the dose (a), tap water (t), plasma sample after dosing (s), and plasma baseline (p) (Ebine *et al*, 2000).

5.2.4 STATISTICAL METHODS

Statistical analyses involved the use of GraphPAD Prism (V5.0, GraphPAD Software, San Diego, CA) and Statistica software (StatSoft Inc, Tulsa, OK, USA). Paired t-tests were used to compare the effect of lower or higher fat diet within each group. Wilcoxon matched pairs t-tests were used to test differences between diabetes and control groups for each diet. Repeated measures 2-factor ANOVA (fasting measures, postprandial area under the curve) or 3-factor ANOVA (postprandial measures) with Bonferroni posttests were used for posthoc comparisons. All relationships between variables were tested using Spearman's rank correlation for non-parametric data. The level of statistical significance was set at a P-value <0.05.

5.3 RESULTS

Only subjects complying with and completing both diets were included in the results (n=7 per group). Details of diet analyses, fasting plasma concentrations of clinical and lipid parameters and postprandial measures of plasma glucose, insulin, triglyceride and VLDL-TG, as well as VLDL-TG fatty acid composition are presented elsewhere (Chapter 4). Briefly, the lower fat diet elicited higher plasma glucose and insulin levels at 2h postprandial in the diabetes group than the lower or higher fat diet at 2h in the control group (Figure 4-2), and also higher fasting glucose levels than lower fat intake by the control group (Figure 4-1A). There was a main effect of diabetes (LF $p<0.002$; HF $p<0.02$) on plasma TG (Figure 4-1E), higher than the control group at fasting, 6h postmeal and 8h postmeal (Figure 4-1). Postprandial plasma TG concentration increased significantly over fasting levels in both groups following both diets and time had a significant effect in the control group when comparing lower fat and higher fat diets ($p<0.02$). Fasting VLDL-TG concentration showed high interindividual variability and was significantly lower following higher versus lower fat intake in control (1.97±1.4 vs 1.15±0.7 mmol/L) but not diabetes subjects (1.57±1.0 vs 2.32±1.5 mmol/L). This included reductions in all fatty acids in fasting VLDL-TG of the control group after 3 days of higher fat intake, significantly for saturated fatty acids, with the general opposite trend occurring in the diabetes group.

5.3.1 PLASMA WATER DEUTERIUM ENRICHMENT

Plasma water deuterium enrichment is shown (Figure 5-1). The one month period in between diets was sufficient to washout ^{2}H enrichment as there was no significant effect of diet order on ^{2}H enrichment of the plasma water. Diets were randomized so that for half of the subjects, the first diet was higher fat and the second diet was lower fat, and vice versa.

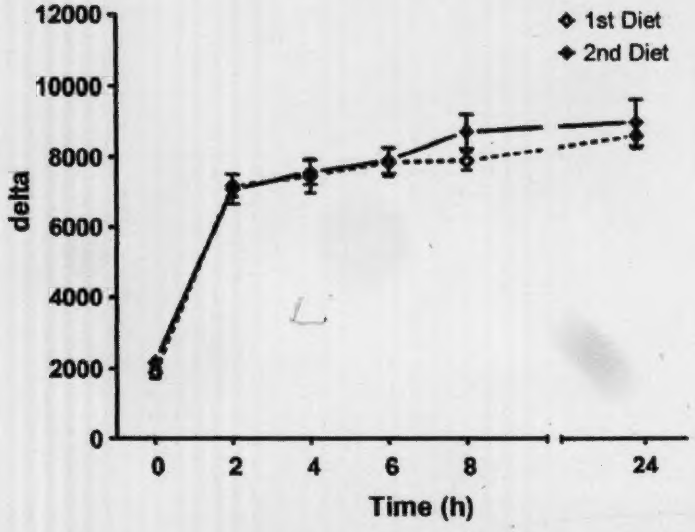

Figure 5-1 Plasma water deuterium enrichment over the study period. Subjects consumed a lower or higher fat diet for 3d (blinded randomized crossover design, 1mo washout). The morning of the third diet day, blood was drawn for determination of 'backround' deuterium enrichment (0h) and immediately a loading dose of deuterium labeled water was ingested followed by a maintenance dose over the following 24 hours (2, 4, 6, 8 and 24h). Values are mean of the total group, error bars = SEM. There was no significant effect of diet order on plasma deuterium enrichment level.

5.3.2 FASTING FATTY ACID SYNTHESIS

A significant effect of diet on the % de novo synthesized fatty acid in VLDL TG after an overnight 12h fast as assessed by the deuterium incorporation method is shown (Figure 5-2A).

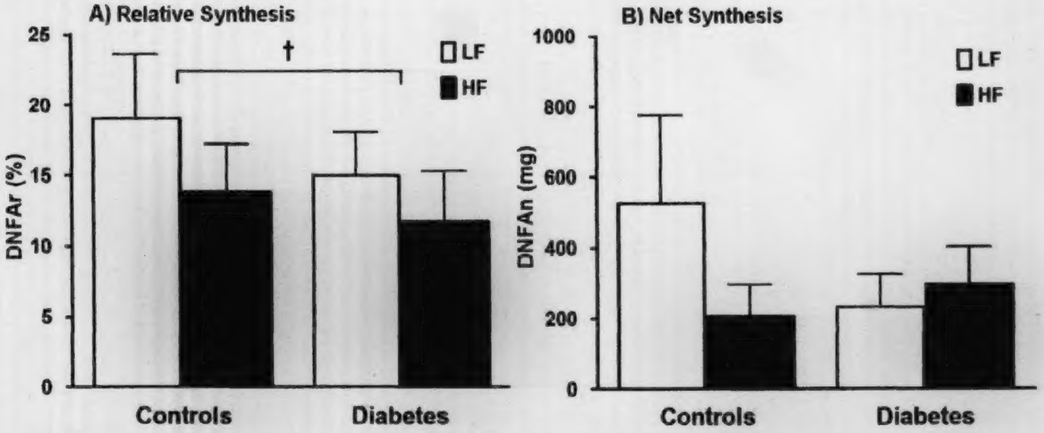

Figure 5-2 Fasting total fatty acid synthesis in VLDL-triglyceride. A) Relative and B) net do novo fatty acids were measured in control and diabetes subjects following 3 days of lower (LF) and higher (HF) fat intake. Values are means with SEM error bars, † indicates a significant main effect of diet at $P<0.05$ as determined by repeated measures ANOVA. No other significant differences were detected.

Relative de novo fatty acid synthesis ranged from 3-33% and 5-25% in control subjects and 2-28% and 0-25% in diabetes subjects following lower and higher fat diets, respectively. Net synthesis ranged from 19-1925mg and 13-739mg in control subjects and 69-778mg and 0-705mg in diabetes subjects following lower and higher fat diets, respectively.

No significant differences found between groups or diets for either relative or net synthesis of any fatty acid (Figure 5-3).

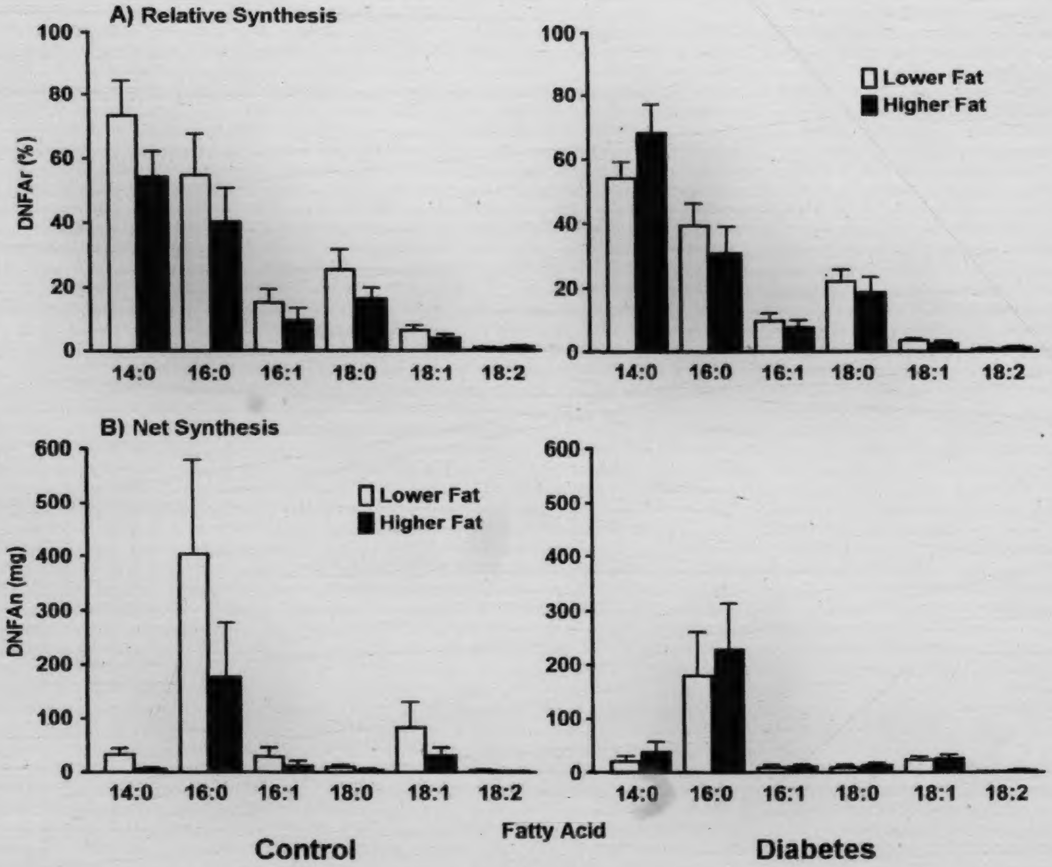

Figure 5-3. Fasting VLDL-triglyceride fatty acid synthesis. A) Relative synthesis (DNFAr) and B) net synthesis (DNFAn) of individual fatty acids in VLDL-TG of control (left panel) and diabetes (right panel) subjects following lower or higher fat intake for 3 days. Bars = mean value, error bars = SEM. There were no significant differences found between diets or groups. For both diets/groups n=7 except n=4 for 14:0 higher fat, and diabetes group 18:0 higher fat n=6, control group 18:0 higher fat n=5 and lower fat n=6.

5.3.3 POSTPRANDIAL FATTY ACID SYNTHESIS

There was a significant main effect of diet (P=0.018) on % de novo rate of synthesis of fatty acids in postprandial VLDL-TG. However, there was also a significant time x diet interaction (P<0.0001). Posthoc analyses indicated that for control subjects, lower fat and higher fat diets differed at 2 hours postmeal (P=0.001)(Figure 5-4A). When area under the curve

(AUC) was calculated for each subject, there was a significant main effect of diet (P=0.02) and a diet x diabetes interaction (P=0.03). Posthoc analyses indicated a difference in relative fatty acid synthesis postprandial AUC between the lower and higher fat diets for the control group (P=0.02)(Figure 5-4A inset). For net postprandial fatty acid synthesis, the only significant effect was a time x diet interaction (P=0.0005), no differences were evident for postprandial AUC measure of net fatty acid synthesis.

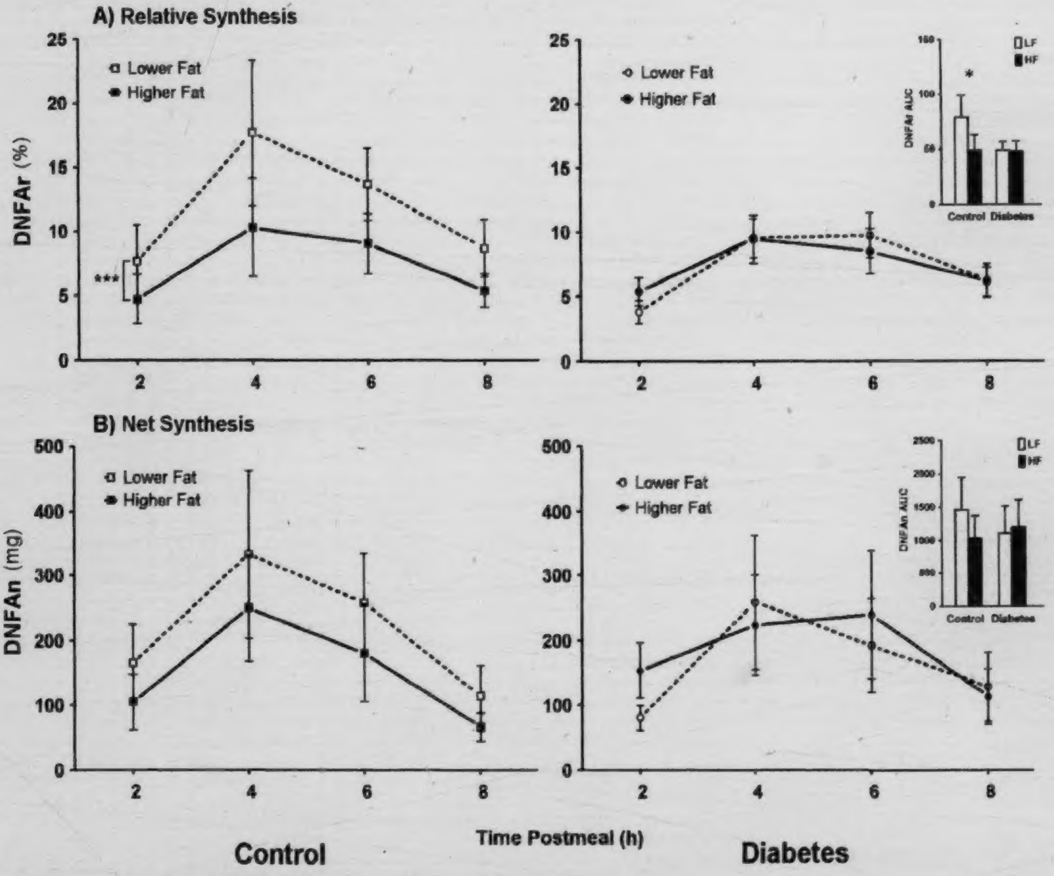

Figure 5-4. Postprandial de novo fatty acid synthesis. A) Relative synthesis (DNFAr) and B) net synthesis of total fatty acids in VLDL-TG of control (left panel) and diabetes (right panel) subjects following 2 days of lower or higher fat intake and then a test meal of the same composition. Values = mean, error bars = SEM. Inset figures: bars are means of area under the curve (AUC) calculations for each subject. * (P<0.05) and *** (P<0.001) represent a significant difference between lower and higher fat diet.

The postprandial data for both total relative % of de novo fatty acid synthesis (DNFAr) and total net synthesis (DNFAn) showed a wide range of interindividual variation. Relative synthesis, or the % of de novo fatty acids in the total pool of VLDL-TG fatty acids, ranged from 0.4-48% and 0.03-29% in control subjects and 0.6-15% and 0.3-15% in diabetes subjects following lower and higher fat diets, respectively. Minimum relative synthesis for both groups was observed at 2h, whereas maximum relative synthesis for control subjects was observed at 4h and diabetes subjects at 6h after the test meal. Net synthesis ranged from 4-624mg and 0.4-519mg in control subjects and 10-851mg and 1-440mg in diabetes subjects following lower and higher fat diets, respectively. Minimum net synthesis for both groups and diets were observed at 2h, whereas maximum relative synthesis was observed at 6h for both groups and diet, except diabetes lower fat. The maximum for this group occurred in the subject at 6h after the lower fat test meal.

Individual fatty acids synthesized during the postprandial period also showed high variation, but some main effects were observed. There was a significant diet effect on relative palmitate (P=0.008) and palmitoleate (P=0.04) synthesized as a percent of total palmitate and palmitoleate in VLDL-TG, respectively. There was also a trend toward an effect of diet on relative oleate synthesized as a % of the total oleate pool (P=0.08). However, there were significant time x diet interactions for these fatty acids (P<0.0001, P=0.004 and P<0.0001, respectively) and a significant time x diet x diabetes interaction for percent de novo oleate (P=0.03). Posthoc analyses revealed that these differences were significant between diets for palmitate and oleate synthesis at 2h postmeal, and oleate synthesis differed between diabetes subjects after lower fat intake and control subjects after higher fat intake (Figure 5-5). Area under the curve was calculated for postprandial data for each subject and there was a trend (P=0.052) for a main effect of diabetes detected for relative rate of oleate synthesis. Posthoc analyses indicate a difference in oleate synthesis postprandial AUC between control and diabetes subjects following lower fat intake (P=0.045)(Figure 5-5D, inset).

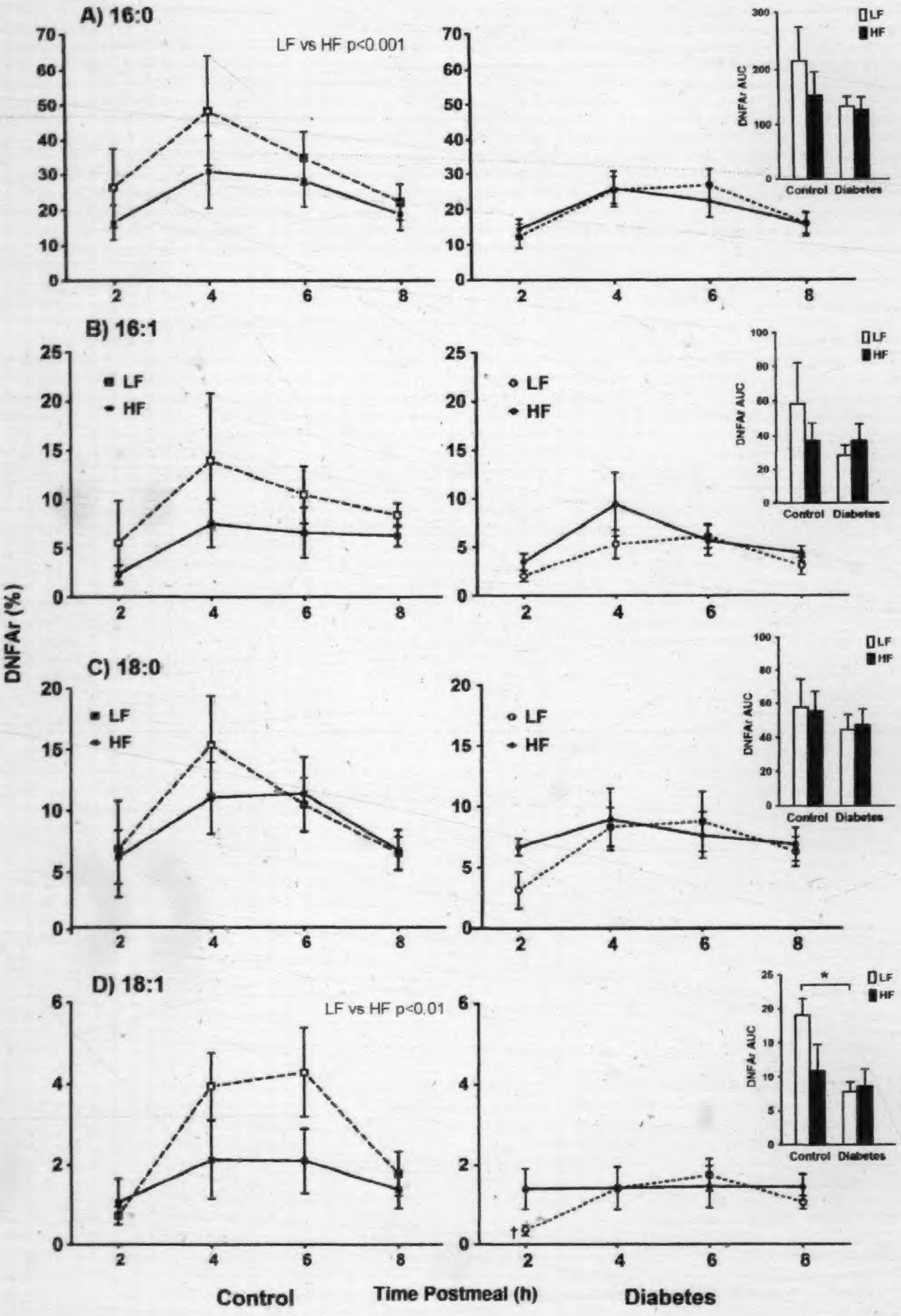
A) 16:0
LF vs HF p<0.001
B) 16:1
LF
HF
C) 18:0
LF
HF
D) 18:1
LF vs HF p<0.01
†
*
DNFAr (%)
DNFAr AUC
LF
HF
Control
Diabetes
2
4
6
8
Control
Time Postmeal (h)
Diabetes

Figure 5-5. The rate of synthesis of each fatty acid in VLDL-TG. The percent of A) palmitate (16:0), B) palmitoleate (16:1), C) stearate (18:0) and D) oleate (18:1) synthesis relative to the total pool of that fatty acid by control (left panel) and diabetes (right panel) subjects following 2 days of lower (LF) or higher fat (HF) intake and then a test meal of the same composition. Values = mean, error bars = SEM. Inset figures: bars are means of area under the curve (AUC) calculations for each subject. * represents a difference between the control and diabetes group for the lower fat diet ($P<0.05$). † represents a significant difference from the 2h control group higher fat value. LF vs HF values represent a significant main effect of diet.

Relative rate of 14:0 synthesis during the postprandial period was also measured, but in many subjects the concentration was too low to get a reliable estimate of ^2H enrichment on the mass spectrometer. Therefore, with the small n, a valid comparison was not possible because of missing data points. In the subjects where 14:0 enrichment was measurable, the relative synthesis as a percent of the total 14:0 pool ranged from 13(2h) -72(4h)% and 0.6(4h)-70(2h)% in control subjects and 9(2h)-63(4h)% and 4(6h)-48(6h)% in diabetes subjects following lower and higher fat diets, respectively.

5.3.4 RELATIONSHIPS INVOLVING VLDL-TRIGLYCERIDE FATTY ACID SYNTHESIS

Fasting TG concentration and other clinical measures with fatty acid synthesis

There were no relationships found between VLDL-TG fatty acid synthesis and fasting plasma triglyceride level for either total amount or the % of each fatty acid synthesized. There were also no correlations between total net VLDL-TG fatty acid synthesis change or % change with diet and fasting plasma TG change or % change. The only relationships of note for fatty acid synthesis with any clinical measure was a strong positive relationship between the relative synthesis of both total fatty acid and 16:1 with BMI following higher fat intake and between 18:0 synthesis and HOMA following higher fat intake.

VLDL-triglyceride fatty acid composition and synthesis

The relationships between fatty acid concentrations (Chapter 4) and relative amounts of fatty acids synthesized in VLDL-TG were more numerous and stronger when subjects consumed the lower fat versus the higher fat diet, as outlined below.

Following higher fat intake, no correlations were found between total VLDL-TG fatty acid concentration, or any fatty acid and the relative synthesis of total or each fatty acid. The only correlation observed was between the both myristic (14:0) and palmitoleic (16:1) acid concentration in VLDL-TG and relative palmitic acid synthesis (16:0).

Following lower fat intake, the only fatty acid concentrations correlated with the relative rate synthesis of the same fatty acid were 14:0 and 16:0. The concentration of 14:0 was also correlated with 16:0 synthesis and 16:0 concentration with 14:0 synthesis. Overall, for the lower fat diet, 14:0 concentration was related to the percent synthesis of all fatty acids measured, including total VLDL-TG fatty acid synthesis. Alternatively, total VLDL-TG fatty acid concentration was correlated with the relative synthesis of 14:0. 18:0 concentration was correlated with both 14:0 and 16:0 synthesis.

Relationship between total percent fatty acids synthesized and total net fatty acid synthesis

In order to determine if any individual fatty acid can be used as an indicator of total fatty acid synthesis, the net fatty acid synthesis (DNFAn as shown in Figure 5-5B) of individual fatty acids was used to calculate the percent contribution of each to total net fatty acid synthesis (Figure 5-2B). There were two fatty acids in which the proportion of synthesis of that fatty acid was correlated with total net fatty acid synthesis. Palmitic acid was positively correlated for both diets (lower fat R=0.69, P=0.0095; higher fat R= 0.57, P=0.044) and oleic acid was negatively correlated for both diets (lower fat R=-0.76, P=0.0027; higher fat R= -0.73, P=0.0041).

5.4 DISCUSSION

It was previously demonstrated that short term higher fat intake in non-diabetic control subjects resulted in a reduction in VLDL-triglyceride compared to eucaloric lower fat intake (Chapter 4). This reduction was primarily due to a decrease in myristic, palmitic and palmitoleic acids, even though levels of these fatty acids were consistent between diets. This effect was not observed in matched diabetic subjects consuming the same lower and higher diets. It was speculated that lipogenesis may be suppressed by higher fat intake in the non-diabetic subjects in the short term. The present study used deuterium incorporation techniques in these subjects in order to determine total VLDL-TG synthesis in response to a change in fat intake. Overall, diet had an effect on VLDL-TG fatty acid synthesis and this effect was more pronounced in the non-diabetic control subjects following lower fat intake. This was likely due to a consistently higher synthesis of individual fatty acids in VLDL-TG, although because of high interindividual variation and the small number of subjects in each group, this sometimes failed to reach statistical significance.

The increase in hepatic fatty acid synthesis as observed following lower fat intake in control subjects in this study is in agreement with previous research showing this effect of diet. Fatty acid synthesis varied greatly between individuals, with a range of 0 to 33% VLDL-TG fatty acids at 12 hours fasting and 0.03 to 48% during the second and fourth hour of the postprandial period, respectively. Lower fat intake consistently increased lipogenesis, which seemed to be upregulated in the postprandial period. Interestingly, this effect was seen following lower fat even though the diet fed was low glycemic index and high in fibre. Previous studies have shown that low fat/high carbohydrate diets that are also low in glycemic index and high in fibre do not have the stimulatory effect on lipogenesis of high carbohydrate/high simple sugar diets. Also, contrary to the hypothesis that both lower fat intake and diabetes would result in upregulation of fatty acid synthesis, particularly palmitic acid, this effect was not seen in diabetes subjects consuming the lower fat diet.

Much of the research surrounding de novo lipogenesis examines the process in young normal weight subjects in the fasting state. The findings of such research resulted in some speculation as to the significance of the contribution of de novo lipogenesis to plasma TG or VLDL-TG concentration. Estimates of the relative synthesis rate of fatty acids synthesized in VLDL-TG from this research were about 4% and it was concluded that de novo lipogenesis was a minor contributor and that the main source was plasma free fatty acids. Further research using combined isotopic techniques determined that during fasting, an average of 4.0±3.6% of VLDL-TG originated from lipogenesis, whereas 77.2±14.0% was from adipose fatty acid release. During feeding the contribution of fatty acids changed on average, with a wide variation in the data as indicated by the high standard error shown. It was determined that the proportion of fatty acids from lipogenesis increased to approximately 8.2±5.1% of

VLDL-TG, 43.6±18.6% came from adipose tissue fatty acids, 15.2±13.7% from chylomicron-remnant TG and 10.3±6.9% from lipolysis of dietary fatty acids into the plasma free fatty acid pool (Barrows & Parks, 2006). These estimates are somewhat lower for lipogenesis than data from the current study would suggest, with both peak and fasting averages for the control group consuming lower fat at around 20% of VLDL-TG. This could be due to the older age and higher BMIs of these subjects, the method of determination of relative synthesis or the difference in diet and/or feeding design of the study.

Indeed, research involving subjects with abnormalities in fat metabolism show that the contributions of lipogenesis can be substantially higher, even in the fasting state. In patients with non-alcoholic fatty liver disease it was shown that the contribution of hepatic lipogenesis to VLDL-TG was higher than controls. Controls had relative rates of lipogenesis rates of 4.6±1.1%, whereas patients had a rate of 14.9±2.7 (Diraison *et al*, 2003). Another study found that even during a high-fat, low-carbohydrate diet, hyperinsulinemic obese subjects had a 3.7–5.3 fold higher rate of lipogenesis than normoinsulinemic obese or lean subjects (8.5 ± 0.7% vs 2.3 ± 0.3% vs 1.6 ± 0.5%). However, after low-fat, high-carbohydrate consumption, rates of lipogenesis were high in all groups, at a rate of around 13% (Schwarz *et al*, 2003). This indicates that both disease state and diet have important roles in the lipogenic rates of individuals.

To put the present results into perspective, a crude estimation of fatty acid synthesis by individual subjects can be examined. Research suggests that VLDL TG production rates for normal subjects is about 200mg/kg/day and up to an average of 400 mg/kg per day (Grundy *et al*, 1979;Olefsky *et al*, 1974). At an average weight for the subjects in this study of 92.7kg, that would equal approximately 19 to 37 g/d of fat being transported throughout the body by VLDL, up to 48% of which could potentially be synthesized by the liver. The value 48% was the highest relative synthesis of all the subjects in this study, as observed in one subject 4 hours after the lower fat test meal. This subject's total relative synthesis ranged from 48% at 4h and 12% at 8h after meal. To examine this particular subject more closely, the average relative synthesis of all measures (fasting and 2, 4, 6 and 8h postmeal) was 26%. At a weight of 76.4kg and VLDL-TG production rates estimated above, this subject could potentially synthesize an average of 6g of fatty acid per day. At a minimum relative synthesis of 12% at 8h and considering it at the lower end of VLDL-TG production (200mg/kg/day), the result would be 2g of fatty acid synthesis per day, whereas at the maximum of 48% synthesis at the higher end of VLDL-TG production (400mg/kg/d), the resultant amount of fatty acids synthesized could be estimated at 15g per day. According to calculations based on net synthesis of each fatty acid at fasting for this subject, 6% of total fatty acids synthesized were 14:0, 82% were 16:0 and 3% were 18:0, for a total proportion of saturated fatty acids of 91% and therefore a potentially daily maximum amount of saturated fatty acids in VLDL-TG per day of 14g. By contrast, during higher fat intake this subject had an average relative synthesis rate of of 18% of total VLDL-TG fatty acid, which at fasting this pool of synthesized fatty acids comprised 68% palmitic acid and 77% total saturated fatty acids. For a subject on the other end of the spectrum, the higher fat diet completely suppressed fat synthesis. Deuterium enrichment in the fatty acids of VLDL-TG in this subject barely increased over baseline. At 24h, the deuterium in all fatty acids except 18:0 had returned to background levels. Fasting total net synthesis for was calculated as 0 following higher fat intake and 69mg after lower fat intake. Using the same calculations as above, at a relative synthesis rate of 3%, the amount of fatty acids synthesized would still be less than 1g per day following lower fat intake.

The differences observed between dietary FA composition and VLDL-TGFA composition is often attributed to the effect of macronutrient composition on de novo lipogenesis, whether it was assumed, concluded due to an indirect measure (Forsythe *et al*, 2008) or determined using stable isotopes (Aarsland & Wolfe, 1998). When diets are lower in fat and higher in carbohydrate, the effect on VLDL-TG fatty acid composition (and some phospholipids and cholesterol esters) tends to be a decrease in saturated fatty acid composition and an increase in linoleic acid. Since it is generally known that palmitate is the most abundantly synthesized fatty acid, it follows that a lipogenesis-inducing diet should influence the types of fatty acids contributed to and therefore the composition of fatty acid in the VLDL-TG.

Results from this study would indicate that using palmitoleate (16:1n-7) levels as an indicator of fatty acid synthesis, as a surrogate marker for elongation/desaturation of 16:0 (Forsythe, 2008) may not be accurate in this study. VLDL-TG 16:1 concentration was not correlated with total net fatty acid synthesis during the fasting state. Further, studies that measure palmitate synthesis and equate it to total hepatic fatty acid synthesis may be warranted as there was a strong positive correlation found regardless of diet. However, it should be noted that there may be an underestimation of synthesis in some subjects using these methods, because according to this research, synthesized palmitate can be as low as 44% of total synthesized VLDL-TG fatty acids.

5.5 CONCLUSION

Much research in the area of lipogenesis has been done using diets that tend to be extreme regarding either the form (liquid vs solid, ingested vs feeding tube) or the macronutrient content (high simple carbohydrate, overfeeding carbohydrate, very high in fat). The intent of the current study was to test the effect of a mixed diet within the usual range of fat and carbohydrate intake. The higher fat diet fed was 10% higher in energy from fat than the lower fat diet in order to suppress lipogenesis, and this diet change had an effect of on 24h fasting fractional synthetic rate of fatty acids in VLDL-triglyceride. Fatty acid synthesis was upregulated in non-diabetic control subjects following short term lower fat intake, despite the low glycemic index/high fibre content of this diet. Estimates of fasting and postprandial lipogenesis showed that the average relative contribution of de novo fatty acid to the total VLDL-triglyceride pool ranged from 4 to 19% of total VLDL-triglyceride; however the rate of synthesis of total and individual fatty acids showed a wide range of variation, between diets and between subjects. Since the major fatty acid product of de novo lipogenesis was palmitate, this could potentially produce an excess of saturated fatty acid in the VLDL-TG of certain individuals. On average, 68% of all de novo fatty acids were palmitate and palmitate synthesis was related to total hepatic fatty acid synthesis. However, research measuring only palmitate synthesis as fatty acid total synthesis should consider that in some cases this may be an underestimation.

Lipogenesis, which is greatly upregulated in some individuals, produces fatty acids that are more saturated, potentially affecting the overall composition of triglyceride in the VLDL. It appears that dietary fat amount is an important determinant of changes in de novo lipogenesis; however in diabetes subjects this effect may be blunted. The effects of variations in synthesis of total and saturated fatty acids in individual subjects may be of particular importance to risk of metabolic disease. Further research will need to determine whether these differences in lipogenic capacity contribute to hypertriglyceridemia and other metabolic alterations that occur in diabetes.

5.6 REFERENCES CITED IN CHAPTER 5

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CHAPTER 6

THE EFFECT OF DIETARY CHANGE OF FAT AND CARBOHYDRATE ON PLASMA FREE FATTY ACID COMPOSITION IN DIABETIC AND NON-DIABETIC SUBJECTS.

6.1 INTRODUCTION

Hypertriglyceridemia is the most common dyslipidemia in diabetes and is considered an independent risk factor for cardiovascular disease (Austin, 1999;Hokanson & Austin, 1996;Sprecher, 1998). Elevated plasma triglyceride (TG) concentration indicates an excess of serum triglyceride-rich lipoproteins, the sources of which are *de novo* lipogenesis, chylomicron triglycerides and plasma nonesterified fatty acids.

Circulating free fatty acids are mostly complexed to albumin and are derived from adipose tissue TG lipolysis, TG-rich lipoprotein lipolysis (chylomicrons, VLDL) or absorbed free fatty acids (Sparks & Sparks, 1985). These circulating fatty acids have an effect on hepatic processes and research suggests that free fatty acid flux into the liver and the rate of re-esterification to TG are major determinants for TG synthesis (Malmstrom *et al*, 1997b). Hence, a reduction of plasma free fatty acid levels would reduce the availability of free fatty acid to the liver as a driver of VLDL synthesis. Originally it was thought that the primary effect of insulin resistance on plasma TG level was a resistance of the anti-lipolytic effect of insulin on adipose tissue. It was more recently been suggested that under normal circumstances, insulin inhibits VLDL production by both free fatty acid-related and free fatty acid independent mechanisms. It is now well-accepted that non-esterified (free) fatty acids not only stimulate VLDL synthesis and secretion (Lewis *et al*, 1995) but are likely the main substrate for VLDL-triglyceride synthesis (Byrne *et al*, 1991)(Barrows & Parks, 2006;Parks *et al*, 1999). It should be noted, however, that much of the research surrounding plasma free fatty acids has involved either in vitro techniques using hepatocytes/cell lines

(Bostrom *et al*, 1988) or in vivo methods utilizing infusions of Intralipid (Lewis *et al*, 1995). In either case, the concentration of fatty acid is often unphysiological (i.e. the presence versus the absence of lipid) and/or the fatty acid composition does not reflect physiological composition (i.e. primarily oleate).

According to isotope tracer studies, free fatty acids from adipose tissue lipolysis during fasting can amount to approximately 77% of VLDL-TG fatty acids, whereas approximately 4% are derived from de novo lipogenesis. In the fed state, these sources seem to switch, attributed primarily to the presence of high insulin. As free fatty acid lipolysis from adipose tissue decreases, so does the contribution to VLDL-TG. Hence, following a meal, the contribution of plasma free fatty acids derived from adipose tissue lipolysis may decrease to 44%, whereas de novo lipogenesis doubles to 8% of fatty acids in VLDL. Other sources make up the remainder of the change postprandially, including 22% dietary fatty acids from hepatic uptake of chylomicron remnant TG and fatty acid spillover from lipoprotein lipolysis into the plasma free fatty acid pool (Barrows & Parks, 2006). Previous research examining the contribution of plasma free fatty acids to VLDL-TG synthesis did not differentiate between adipose-source and diet-source (Parks *et al*, 1999).

Interestingly, research investigating fatty acid release from the adipose tissue of obese subjects showed continual free fatty acid mobilization even under conditions when it should be completely suppressed (Coppack *et al*, 1992). Therefore, both adipose tissue insulin resistance and hepatic insulin resistance may have roles in the elevated plasma free fatty acid levels that occur in type 2 diabetes. Alternatively, elevated plasma free fatty acids are thought to induce insulin resistance. The type of fatty acid available seems to be important because saturated fatty acids appear to cause insulin resistance whereas unsaturated fatty acids seem to be protective or even improve insulin sensitivity (Clandinin *et al*, 1993). In the case of dietary fatty acids, high saturated fat intake results in more saturated membrane lipids (Vessby, 1995). More recently, it was shown in vitro that saturated fat induced skeletal muscle insulin resistance by stimulating intramyocellular accumulation of fatty acyl-CoA metabolites (Adams *et al*, 2004). The mechanism is not completely understood, however saturated fats are less readily oxidized and accumulate as diacyglycerol and ceramide, whereas mono- and polyunsaturated fats accumulate as intramyocellular TG or free fatty acids (Lee *et al*, 2006). Despite these indications of the importance of free fatty acid composition to insulin resistance, research doesn't generally take into account the individual fatty acids that comprise this pool when investigating lipid metabolism.

Plasma free fatty acids not taken up for use by the body are cleared by the liver, potentially exerting direct effects on hepatic processes. Fatty acid structure may be an important determinant of fatty acid regulated nuclear receptor activity (Pawar & Jump, 2003). Studies by Pawar and Jump using rat primary hepatocytes indicated that several factors may control hepatic levels of PPAR ligands including lipogenic and peroxisomal beta-oxidation enzyme activities, as influenced by non-esterified fatty acid composition (Pawar & Jump, 2003). The intracellular non-esterified fatty acid pool is affected by fatty acid entry and exit (i.e. fatty acid transporters, synthesis, oxidation) and so free fatty acids entering the liver can influence the fatty acid composition of stored hepatic triglyceride and fat metabolism in the liver.

It is known that both plasma free fatty acids and hepatic de novo lipogenesis contribute to VLDL-TG synthesis. It is thought that free fatty acids are the primary quantitative contributor to VLDL-TG, however the influence of fatty acid composition on either VLDL-TG composition or concentration has not been examined. Further to this, it is not known whether the deuterium incorporation techniques used to estimate hepatic de novo lipogenesis actually include some label from the plasma pool. Since this pool is a quantitative contributor, the qualitative aspect requires investigation. And finally, it is important to examine if plasma fatty acid composition varies considerably between individuals and is dependent on dietary factors and if this is related to other aspects of lipid metabolism.

The purpose of this research was to alter the carbohydrate and fat intake in subjects with diabetes versus non-diabetic subjects and examine the effect on fasting and postprandial plasma free fatty acid levels in order to add to current understanding of diabetes, diet and fat metabolism. The objective was to determine if de novo lipogenesis contributes to the plasma non-esterified pool of free fatty acids and to what extent this may influence the VLDL triglyceride fatty acid composition as well as contributing to the de novo fatty acids present in VLDL-TG fatty acid. Additionally, it was important to determine if free fatty acid composition or synthesis varies depending on dietary fat level or the presence of diabetes/insulin resistance. It is hypothesized that plasma free fatty acid composition is similar to VLDL-triglyceride fatty acid composition as these are the main source of fatty acids for VLDL-triglyceride synthesis. It is speculated that there may be a small contribution of intestinal or adipose tissue de novo fatty acid to VLDL-TG synthesis, and this will be detected in plasma free fatty acids through the release of labeled fatty acids from adipose tissue triglyceride lipolysis (fasting) or spillover from chylomicron triglyceride lipolysis (postprandial).

6.2 SUBJECTS AND METHODS

6.2.1 STUDY PARTICIPANTS

As described in Chapter 4.2.1, 11 subjects with type 2 diabetes and 10 matched non-diabetic (control) subjects were recruited and screened. At the time of screening (at least 1 month prior to study start) the diabetes and non-diabetic control subjects completing the study design and included in the results (n=7 in each group) were similar in age, BMI, waist circumference and fasting measurements of plasma total, HDL cholesterol, TG, insulin, C-peptide, HOMA, creatinine, ALT and alkaline phosphatase. However the diabetes subjects had significantly higher plasma glucose (6.2±1.1 vs 5.0±0.4 mmol/L) and HBA1c (5.9±0.5 vs 5.3±0.4 %), whereas control subjects had higher LDL cholesterol levels (2.67±0.6 vs 3.46±0.7 mmol/L) (Table 4-1). Interestingly, plasma free fatty acids were lower in the diabetes group (0.66±0.3 vs 0.88±0.8 mmol/L), however this difference did not reach significance level.

6.2.2 STUDY DESIGN

All subjects were fed two diets for 3 days in a blinded randomized crossover design separated by a minimum one-month washout period (Chapter 4.2.2). Subjects were tested at the same time each month to account for hormonal variation in the menstrual cycle of the female subjects. Diets consisted of identical items differing in energy from fat (lower fat <25%; higher fat >35% achieved by addition of canola oil)(Table 4-2).

On test day 1 (diet day 3), a 12h fasting (0h) blood sample was drawn as "background" deuterium enrichment. Subsequently, subjects drank a priming dose of ^2H_2O at 1.0g/kg estimated body water (60% of body weight). Immediately following, the test day 'breakfast' meal was consumed containing the same fat and carbohydrate composition as that of the dietary treatment period providing 1/3 of daily caloric intake (~700kcals; Table 4-3). At regular intervals throughout the next 24h, subjects drank a maintenance dose of 1.0 g ^2H2O/kg estimated body water diluted in 1.5L regular bottled water to maintain plasma levels of deuterium at plateau. Postprandial blood was drawn 2, 4, 6 and 8 hours after test meal consumption. The 'lunch' meal was served immediately following the 8 hour fast and a 'dinner' meal was sent home with subjects to be consumed a minimum of 12h before the blood draw the following morning. The final 12h fasting blood sample drawn on test day 2 at ~09:00h and was used in determination of 24h fatty acid synthesis.

6.2.3 ANALYTICAL METHODS

Diet Composition

Composition of background diet and study diet was calculated using Food Processor II nutrient analysis computer software incorporating GLC determination of study oils. Diet composition achieved a mean lower fat intake of 23% and a higher fat intake of 36% of energy from fat (Table 4-3). Test meals were prepared according to calculated recipes and fat was extracted and analyzed to confirm that composition of meals met the diet design for fatty acid composition (Table 4-4).

On test day 1 (diet day 3) of each diet treatment, a fasting blood sample was drawn, the test meal breakfast was consumed and subjects fasted for 8 hours (Chapter 4.2.3). Blood was collected at 2, 4, 6, and 8h, the 2 remaining meals of the diet were consumed a minimum of 12h before the final blood draw on test day 2. VLDL-TG fatty acids were extracted from plasma samples and fatty acid methyl esters were analyzed by GLC as previously described (Chapter 4.2.4).

Plasma Free Fatty Acid Concentration and Composition

Free fatty acids were extracted from plasma and methylated to form fatty acid methyl esters (FAMEs) in a one-step reaction developed by Lepage and Roy (Lepage & Roy, 1988). Briefly, 150 μL of plasma was added to a glass test tube containing a mixture of internal standards (13:0 15μL, 17:0 10μL, 19:0 5μL) dissolved in 5 mL of methanol-acetyl chloride 50:l (v/v). A small magnetic bar was placed in each tube and Teflon-lined caps were closed tightly. Tubes were placed in a stir-plate at 27°C and stirred constantly for 45 minutes. At this time 3 ml of 6% K2CO3 solution was slowly added to stop the methylation reaction. Hexane was then added in the amount of 150 μl and the tubes were shaken and centrifuged at 2000g for 10 minutes. The cloudy upper phase of hexane containing the FAMEs was removed with a 100 μl glass syringe and put into a small GC injection vial with a 200 ul glass insert, capped and placed in a 1 μl was injected into the Varian 3600CX Gas Chromatograph by flame ionization and helium as a carrier gas (1.9 ml/min at 80°C). The split ratio was set at 7:l. The silica column used was a 60-m fused silica column wall-coated with 0.20 pm SP-2331 (25% bonded phase and an internal diameter of 0.32 mm). Samples were run in duplicate and calculations using 17:0 and 19:0 standards for fatty acid quantification were averaged to arrive at concentration values for each fatty acid.

Total Relative and Net Amount of De novo Synthesized Free Fatty Acid

Isotopic analysis of non-esterified fatty acid deuterium (^2H) enrichment was performed by ^2H incorporation techniques using isotope ratio mass spectrometry. The methods detailed in Chapter 5 for ^2H analysis of VLDL-TG and constituent fatty acids were adapted for use with plasma non-esterifed fatty acids after extraction from plasma by Folch/TLC preparation and subsequent methylation.

Briefly, the maximum possible enrichment of VLDL-TG was considered to be equal to the enrichment of plasma after ^2H_2O administration, corrected for the maximum number of ^2H atoms which could enter the newly synthesized fatty acids (Konrad, 1999). The maximum ^2H incorporation was calculated at 0.87 g-atoms ^3H per g-atom C based on adipose fatty acid synthesis (Jungas, 1968). The correction factor for plasma deuterium enrichment can be calculated using the ratio of carbon to hydrogen for the molecule (Leitch & Jones, 1991).

The equation used for free fatty acids there was the same as equation 2 in Chapter 5 without the need to correct for glycerol. This was 0.477 based on the average plasma free fatty acid (17:1), outlined as follows:

$$\frac{0.87\,^{2}H}{C_{fa}} \times \frac{17C_{fa}}{31H_{fa}} = \mathbf{0.477}\,\frac{^{2}H}{H_{fa}} \qquad (1)$$

The ratio between the actual enrichment over 24h and the maximum enrichment possible can be used to represent the relative amount of de novo synthesized fatty acid in the fatty acid pool. In terms of percent, this relationship can be described in the following equation:

$$\text{DNFAr}\ [\%] = \frac{\delta FFA_{ENR} - \delta FFA_{BG}}{(\delta PW_{ENR} - \delta PW_{BG}) \times \mathbf{0.477}} \times 100 \qquad (2)$$

Where ENR represents 'enriched' sample or ^{2}H enrichment after $^{2}H_2O$ administration and BG represents 'background' sample or natural abundance of ^{2}H before administration of $^{2}H_2O$ in the plasma free fatty acid fraction and as measured in the plasma water (PW) portion of the sample for each subject.

An estimate was also made of the net amount of de novo synthesized fatty acid in the free fatty acid pool (DNFAn). This can be calculated from fractional synthetic rate and an estimation of total free fatty acid pool size as follows:

$$\text{DNFAn [mg]} = \text{(FSR)(FFA [mg/L])(plasma vol est[L])} \qquad (3)$$

Where FSR is the fractional synthetic rate (DNFAr expressed as a fraction, equation 3) and free fatty acid (FFA) pool size is estimated from free fatty acid concentration as measured by quantitative GLC analysis and an estimation of total plasma volume. Plasma volume was estimated for each subject at 37.5 ml/kg for females and 45 ml/kg for males (Dagher *et al.*, 1965).

Relative and Net Synthesis of Individual Fatty Acids in Free Fatty Acid Pool

For the synthesis of individual free fatty acids, the above calculations were used with the correction factor adjusted according to the triglyceride representing each fatty acid rather than the average fatty acid. For example, when the fatty acid in question was palmitic acid (16:0), the correction factor was determined as follows:

$$\frac{0.87D}{C_{fa}} \times \frac{16C_{fa}}{31H_{fa}} = \mathbf{0.449}\,\frac{{}^{2}H}{H_{fa}} \qquad (4)$$

The correction factor for each fatty acid was calculated as: myristic acid 0.487, palmitic acid 0.449, palmitoleic acid 0.480, stearic acid 0.477, oleic acid 0.447, linoleic acid 0.505.

Samples were analyzed as outlined in Chapter 5. Briefly, if data was not consistent, samples were reprepared and rerun in duplicate. If the area under the peak for a particular fatty acid was <0.1, the concentration of the fatty acid for the purposes of ^{2}H enrichment calculations was considered to be negligible. When the instrument separated peaks for isomers the 18:1n-7 and 18:1n-9, peaks were manually selected to include both peaks together and considered as total 18:1.

Plasma Water Deuterium Enrichment

Isotopic analysis of plasma water deuterium (^{2}H) enrichment was performed using standard vacuum techniques (Jones *et al*, 1988) as outlined in Chapter 5. Sample ^{2}H enrichment was analyzed using a 903D dual-inlet isotope ratio mass spectrometer (IRMS; VG Isogas, Cheshire, UK). Enriched samples for each subject were diluted dilution with distilled water as previously described (Ebine *et al*, 2000) and all samples were run in duplicate or triplicate.

6.2.4 STATISTICAL METHODS

Statistical analyses involved the use of GraphPAD Prism (V5.0, GraphPAD Software, San Diego, CA) and Statistica software (StatSoft Inc, Tulsa, OK, USA). Paired t-tests were used to compare the effect of lower or higher fat diet within each group. Wilcoxon matched pairs t-tests were used to test differences between diabetes and control groups for each diet. Repeated measures 2-factor ANOVA (fasting measures) or 3-factor ANOVA (postprandial measures) with Bonferroni posttests were used for posthoc comparisons. All relationships between variables were tested using Spearman's rank correlation for non-parametric data with a significance level of $P \leq 0.01$. The level of statistical significance for all other data was set at a P-value <0.05.

6.3 RESULTS

Only subjects complying with and completing both diets were included in the results (n=7 per group). Details of results such as dietary analyses, fasting plasma concentrations of clinical and lipid parameters and postprandial measures of plasma glucose, insulin, triglyceride and VLDL-triglyceride, as well as VLDL-triglyceride fatty acid composition are presented (Chapter 4).

Figure 5-1 demonstrates that by 2h after the loading dose of deuterium oxide there was a rapid increase in plasma deuterium enrichment, which plateaued for the time remaining in the study period. Diet order did not have an effect on plasma water deuterium enrichment.

6.3.1 FASTING MEASURES

Concentration, Composition and Synthesis of Plasma Free Fatty Acids

There was no significant effect of diabetes or diet on fasting plasma free fatty acid concentration following 3 days of lower or higher fat intake (Figure 6-1A). There was a significant main effect of diet on both the relative synthesis (P=0.012, Figure 6-1B) and net synthesis (P=0.027, Figure 6-1C) of total free fatty acids in fasting plasma. Posthoc comparisons revealed a trend towards a difference between lower and higher fat diets for net fatty acid synthesis in the diabetes group (P=0.076).

When the concentration of individual fatty acids was examined, there were no significant main effects of diet or diabetes on any fatty acid (Figure 6-2A). There was a trend towards a diet x diabetes interaction for 16:0 concentration (P=0.08). Paired comparisons showed no significant differences between diets for either group.

There was a main effect of diet on the relative synthesis of 16:0 (P=0.03), 18:0 (P=0.01), 18:1 (0.04) and 18:2 (P=0.04), but posthoc comparisons were not significant. Paired comparisons revealed a significantly lower 18:0 relative synthesis following higher versus lower fat intake (P=0.04)(Figure 6-2B,). Valid statistical analyses could not be done for 14:0 and 16:1 the concentration of these fatty acids were too low in some subject's plasma to analyze deuterium enrichment (as outlined in 6.2.3 Analytical Methods: DNFAr and DNFAn for Individual Fatty Acids in Free Fatty Acid Pool).

There were no main effects of diabetes or diet observed for net synthesis of free fatty acids, except for 18:1, in which there was an effect of diet (P=0.029)(Figure 6-2C). There was a trend towards an effect of diet on 18:2 synthesis (P=0.06). Similarly to relative synthesis, posthoc comparisons revealed no significant differences and 14:0 and 16:1 were omitted from all analyses. It should also be noted that 18:2 is an essential fatty acid and therefore synthesis should equal zero. Any deuterium enrichment in this fatty acid can be considered "noise" in the analysis technique and synthesis above the level of this fatty acid validated as actual synthesis of the fatty acid (Figure 6-2B and 6-2C).

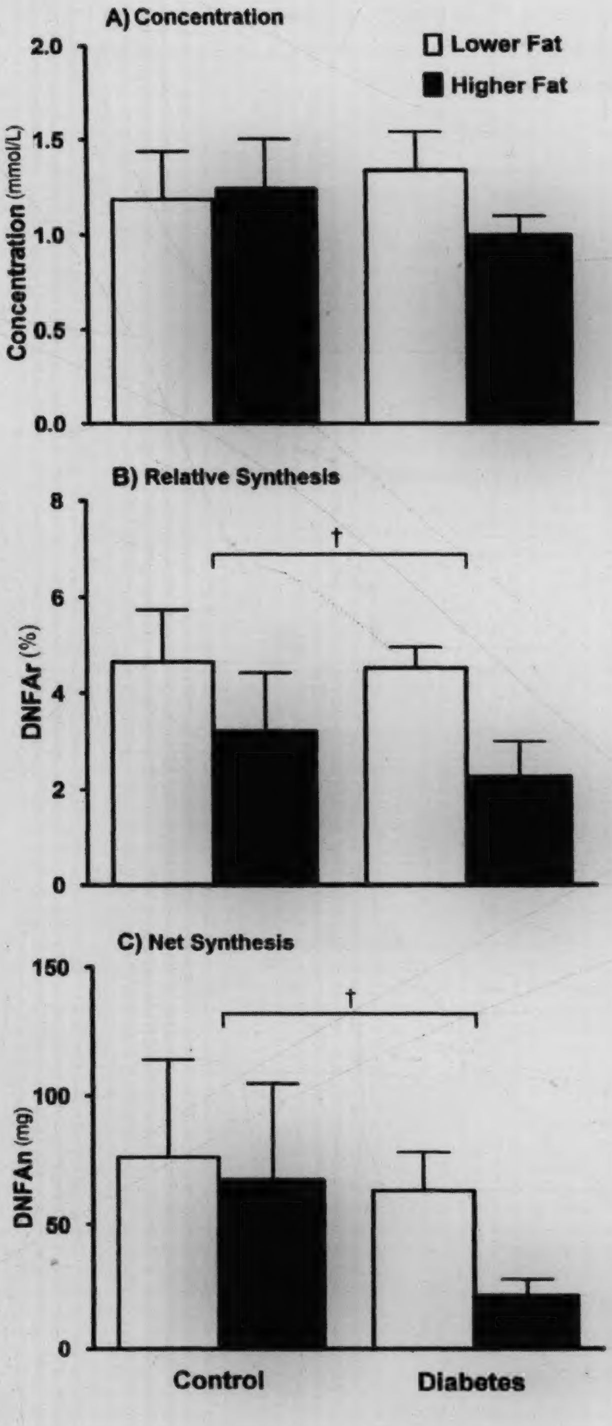

Figure 6-1. Total plasma free fatty acids. A) Concentration, B) relative synthesis (DNFAr) and C) net synthesis (DNFAn) of fasting plasma free fatty acids from control and diabetes subjects following 3 days of lower or higher fat intake. Bars = mean values, error bars = SEM. † denotes a main effect of diet ($P \leq 0.05$) by repeated measures 2-factor ANOVA (diet, diabetes).

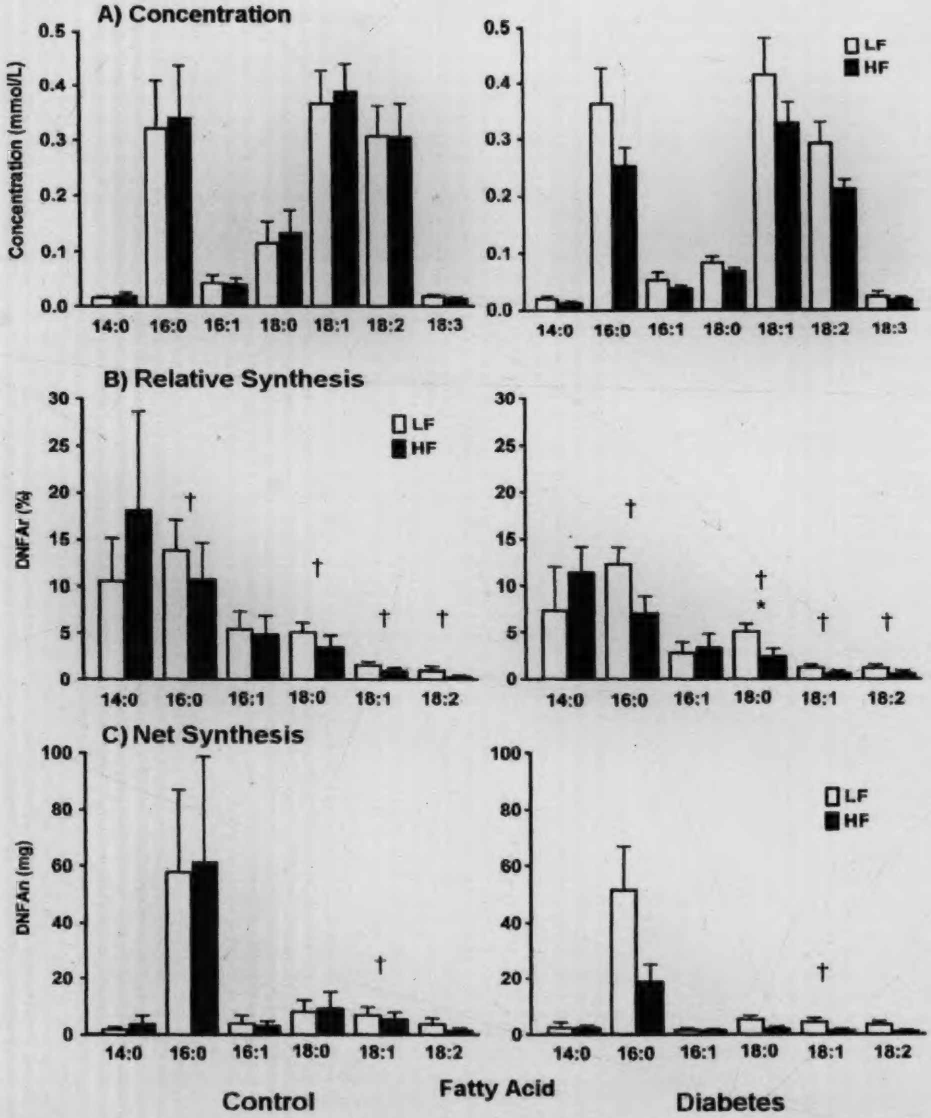

Figure 6-2. Composition of fasting plasma free fatty acids synthesized de novo. A) Control and B) diabetes subjects consumed lower or higher fat intake for 3 days. Values are means, error bars = SEM. * indicates a significant difference between diets (P<0.05) for that group by paired t-test and † denotes a main effect of diet on that fatty acid (P≤0.05) by repeated measures 2-factor ANOVA (diet, diabetes). For DNFAr and DNFAn, n was = 3, 3, 2 and 3 for 14:0 and 5, 5, 3, and 4 for 16:1 (control lower fat, control higher fat, diabetes lower fat, diabetes higher fat, respectively); valid within subject comparisons could not be performed.

6.3.2 POSTPRANDIAL MEASURES

Postprandial Plasma Free Fatty Acid Concentration and Composition

The concentration of total plasma free fatty acids was measured 2, 4, 6 and 8h after a test meal lower or higher fat test meal (Figure 6-3). There were no significant effects of diet or diabetes, but there was a significant effect of time (P=0.0005) on the postprandial curves and a time x diet interaction (P=0.007). Posthoc analyses did not reveal any further significant differences except that fatty acid concentration changed over time.

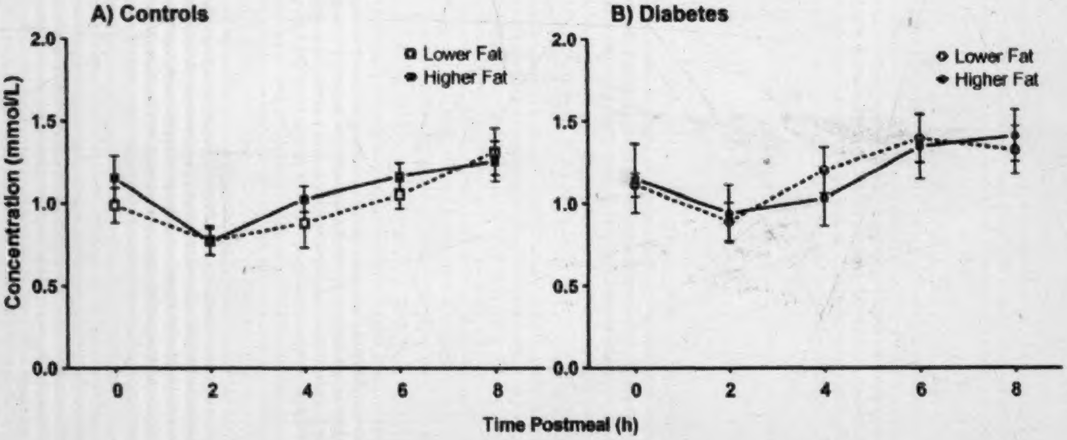

Figure 6-3. Total postprandial plasma free fatty acid concentration. Plasma concentration of free fatty acids in A) control and B) diabetes subjects following 2 days of lower or higher fat intake and then a test meal of the same composition. Values = mean, error bars = SEM. Main effects were time (P=0.0005) and a time x diet interaction (P=0.007).

The concentration of individual free fatty acids was measured following the lower and higher fat test meal. Postprandial curves for the plasma concentration of major (Figure 6-4) and minor (Figure 6-5) free fatty acids are shown. There were no effects of time, diet or diabetes on plasma 18:0 or 18:2 concentrations. However, plasma levels of 14:0, 16:0, 16:1, 18:1 and 18:3 changed significantly over time and both 16:1 and 18:1 showed significant time x diet interactions (Figure 6-5B and 6-4A, respectively). Posthoc analyses revealed the interaction was significant at the same timepoint for 0h (fasting), 2 and 6h for 16:0 and 2 and 4h after the test meal for 18:1.

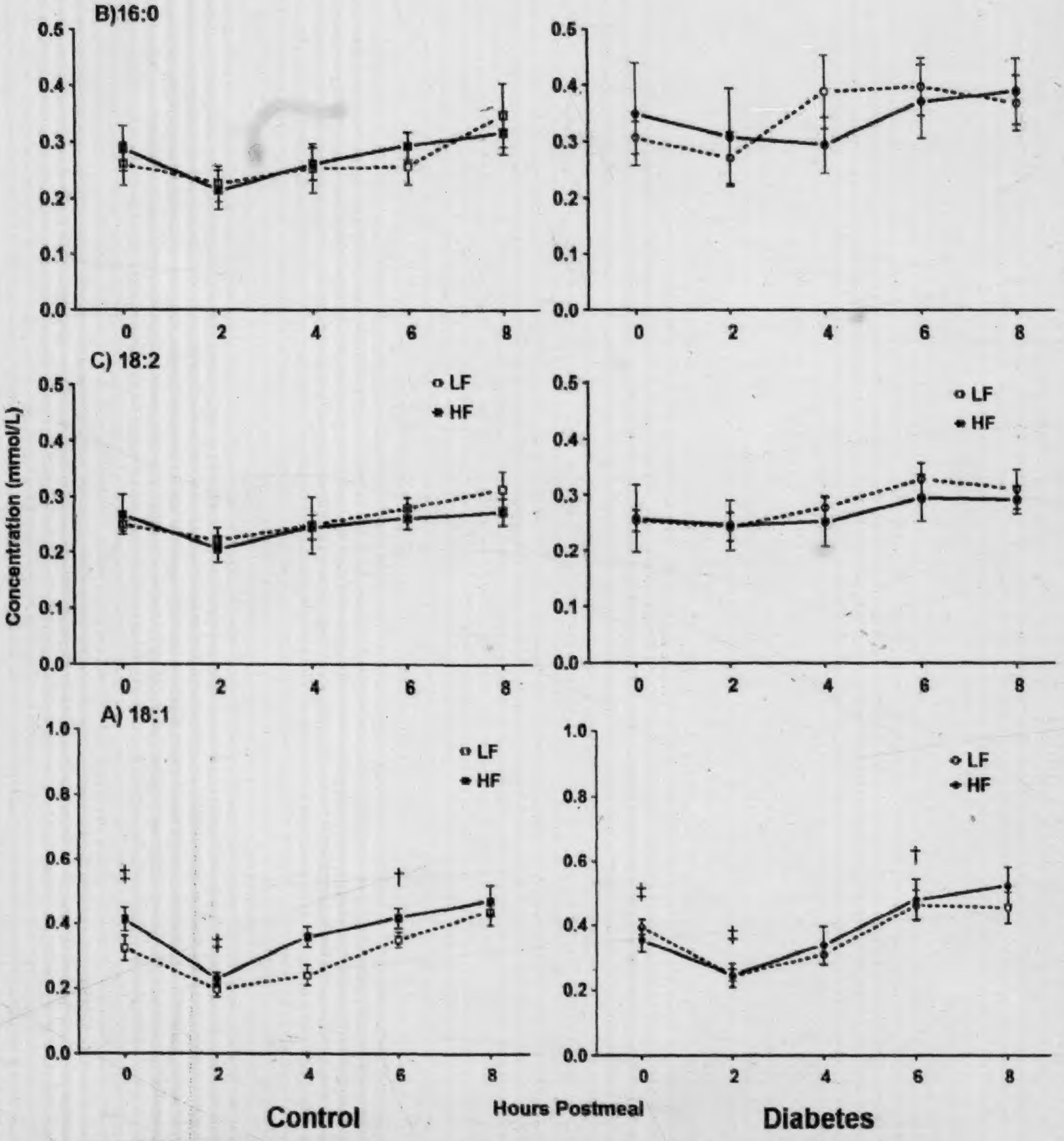

Figure 6-4. Postprandial concentration of major free fatty acids in plasma. A) Palmitic acid (16:0), B) oleic acid (18:1) and C) linoleic acid (18:2) concentrations in control (left panel) and diabetes (right panel) subjects following 2 days of lower or higher fat intake and then a test meal of the same composition. Values = mean, error bars = SEM. Main effects were time for 18:1 ($P<0.000001$) and 16:0 ($P=0.028$) and a time x diet interaction ($P<0.0000001$) whereby † ($P\leq0.05$) and ‡ ($P\leq0.01$) represent diet comparisons at each timepoint where posthoc analysis of the interaction was significant.

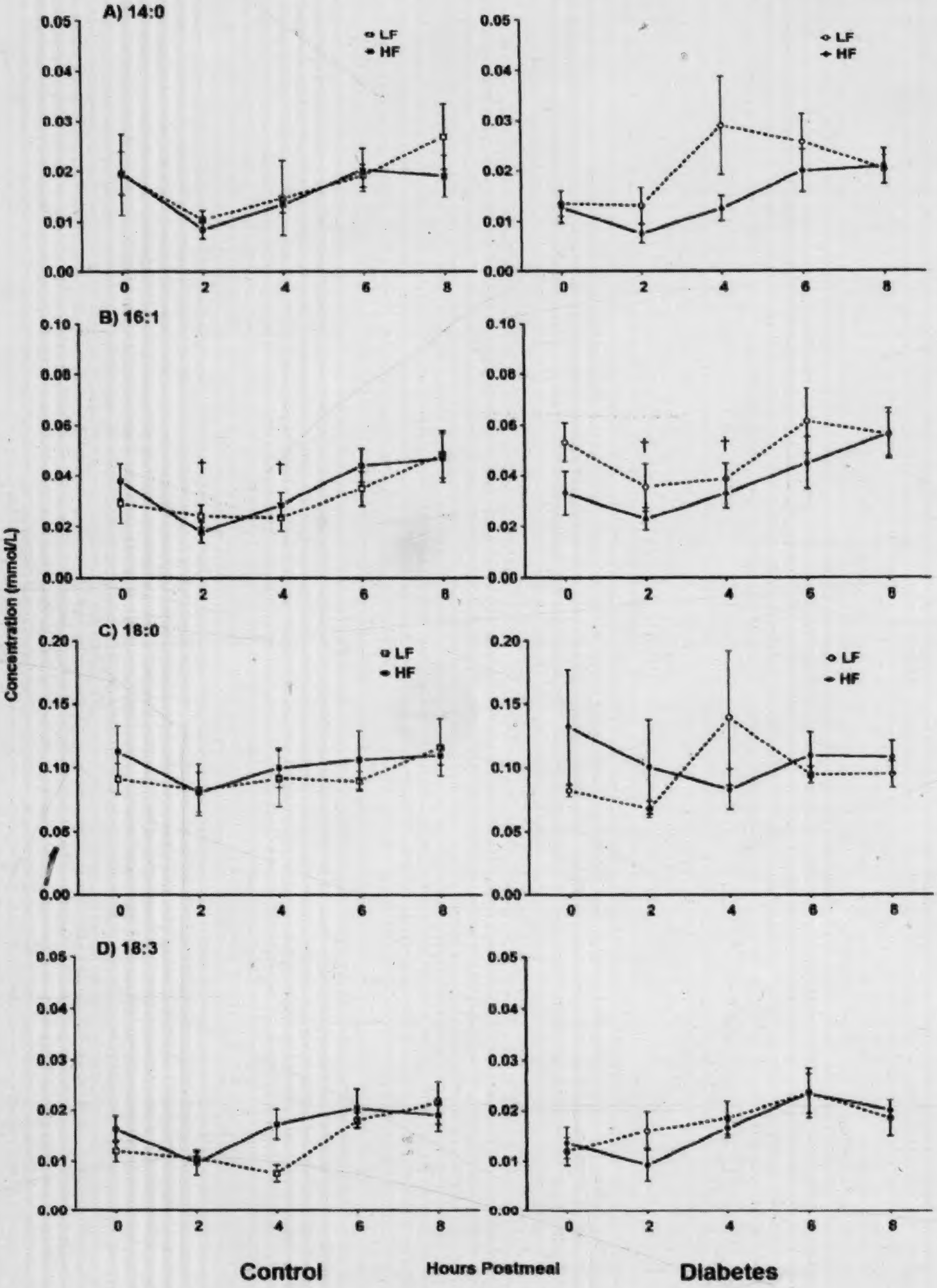
A) 14:0
B) 16:1
C) 18:0
D) 18:3
LF
HF
Concentration (mmol/L)
†
Control
Hours Postmeal
Diabetes
0
2
4
6
8
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.08
0.10
0.15
0.20

Figure 6-5. Postprandial concentration of minor free fatty acids in plasma. A) Myristic acid (14:0), B) palmitoleate (16:1), C) stearate (18:0) and D) α-linoleic acid (18:3) for control (left panel) and diabetes (right panel) subjects following 2d of lower or higher fat intake and a test meal of the same composition. Values = mean, error bars = SEM. Main effects from repeated measures 3-factor ANOVA were time for 14:0 (P+0.002), 16:1 (P=0.0002) and 18:3 (P=0.001). Time x diet interactions were observed for 16:1 (P=0.00001) and 18:3 (P=0.004), where † (P<0.05) represents diet comparisons at timepoints where posthoc analysis of the interaction were significantly different.

Postprandial Free Fatty Acid Synthesis

The relative (DNFAr) and net (DNFAn) synthesis were determined for postprandial plasma free fatty acids (Figure 6-5). There were no significant effects of time, diet or diabetes on the postprandial curves. Although fatty acid synthesis appears to be higher after the lower fat test meal, large individual variation precluded any detection of statistical trends in the data.

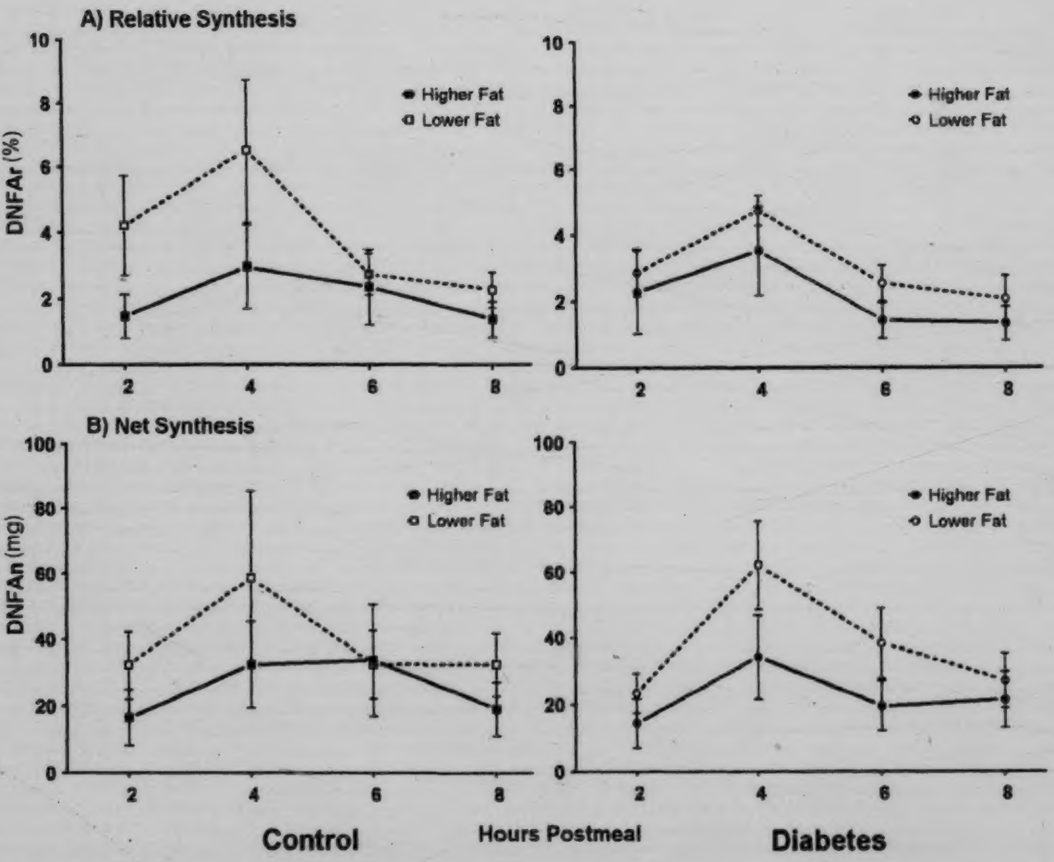

Figure 6-6. Total de novo free fatty acids in plasma in the postprandial period. A) Relative synthesis (DNFAr) and B) net synthesis (DNFAn) of total free fatty acids in plasma of control (left panel) and diabetes (right panel) subjects following 2 days of lower or higher fat intake and then a test meal of the same composition. Values = mean, error bars = SEM. No significant main effects of time, diet or diabetes were detected by repeated measures ANOVA.

The ranges for both the relative and net amount of free fatty acid synthesized varied greatly. For the control group, total net synthesis ranged from 0-208mg and 0-124mg, and for the diabetes group synthesis ranged from 0-124mg and 0-103mg for lower and higher fat test meals, respectively. There was no set pattern for the minimum value as 0 synthesis occurred for many subjects over many different timepoints, however the maximum value usually occurred at 4h postmeal.

The relative amount of synthesis (DNFAr) of individual fatty acids was determined as a percent of fatty acid synthesized in the total pool of that fatty acid. Only 16:0, 18:0 and 18:1 had data sets complete enough to do valid statistical comparisons (n≥6 for each timepoint)(Figure 6-6). There were no significant effects of time, diabetes or diet on the synthesis of plasma 16:0, but time had a significant effect on 18:0 and 18:1 synthesis. There was also a significant effect of diet on 18:0 synthesis, and a time x diet interaction, which posthoc analyses indicated a significant difference for the same timepoint at 2h postmeal (Figure 6-6B).

Postprandial synthesis data was incomplete for 14:0, 16:1 and 18:2 and thus any representation of the data graphically or statistically would be misleading. This is considering that all comparisons are either within subjects (over time or between diets) or between matched subjects (control versus diabetes) and missing data points could have a significant impact on the means. Hence, the data is presented as the ranges of relative synthesis (% of fatty acid pool comprised of synthesized fatty acids) over the postprandial period. For 14:0, relative synthesis ranged from 0-22% and 0-12% for the control group and 0-18% and 0-7% for the diabetes group, for lower and higher fat intake, respectively. For 16:0, synthesis ranged from 0-22% and 0-12% in the control group, 0-18% and 0-7% in the diabetes group, for lower and higher fat, respectively. Ranges observed for 18:2 were 0-4% and 0-1% for the control group, 0-5% and 0-3% for the diabetes group, although greater than half of the timepoints had 0% synthesis. Given that 18:2 is an essential fatty acid that cannot be synthesized by the body, it can be assumed that any deuterium enrichment detected in this fatty acid is associated with "noise" in the analysis technique. It can further be assumed that any deuterium enrichment above this level of noise represents actual fatty acid synthesis.

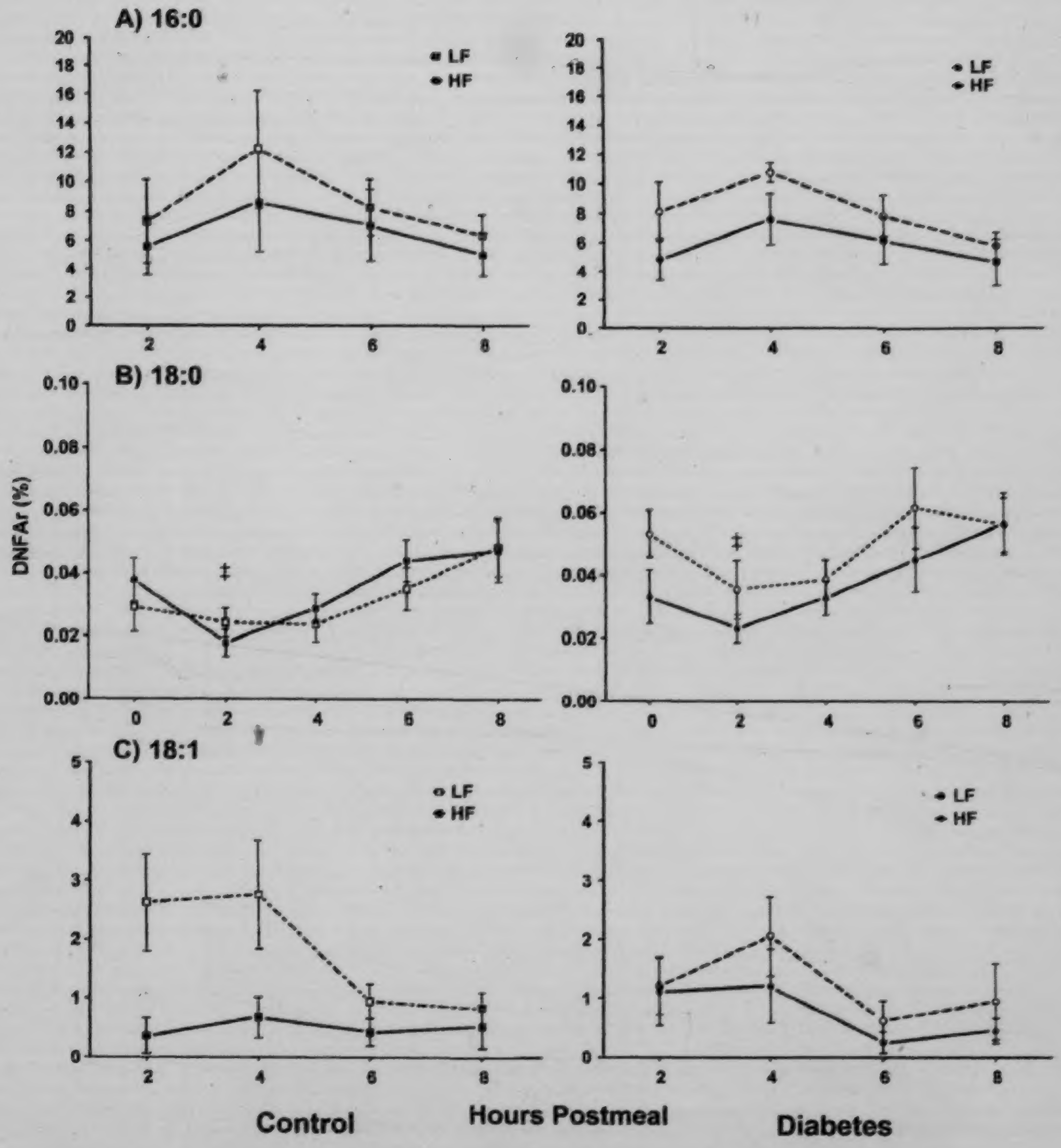

Figure 6-7. Synthesized fatty acids in plasma in the postprandial period. Relative amounts of A) palmitic (16:0), B) stearic (18:0) and C) oleic (18:1) acid in control (left panel) and diabetes (right panel) subjects following 2d lower or higher fat intake and a test meal of the same composition. Values = mean, error bars = SEM. Main effects were: time for 18:1 (P=0.0003) and 18:0 (P=0.008), and diet for 18:0 (P=0.006), and time x diet (P=0.003), where ‡ (P≤0.01) represents diet comparison at the timepoint where posthoc analysis of the interaction was significantly different. Note large differences in scale (x axis).

6.3.3 RELATIONSHIPS INVOLVING DE NOVO SYNTHESIZED PLASMA FREE FATTY ACID

In order to tease out the most important relationships, only correlations with a probability of ≤0.01 were considered significant. Because there were no significant effects of disease on any outcome measured in this study, non-diabetic controls and diabetic subjects were analyzed as one group for all correlations (n=14). Diet data was analyzed separately, as lower fat intake and higher fat intake.

Fasting Triglyceride Concentration and Other Clinical Measures

All clinical results from these subjects are shown in Chapter 4.

There were no relationships found between free fatty acid concentration or synthesis and fasting plasma triglyceride level or VLDL-TG fatty acid concentration. Total free fatty acid concentration and most individual fatty acids were correlated with HOMA for both diets, however, during higher fat intake this only reached significance for VLDL-TG 16:1 concentration (R=0.70, P=0.005). During lower fat intake 16:0 was correlated with HOMA (R=0.76, P=0.002), as well as 14:0 (R=0.80, P=0.0005), and 18:1 (R=0.67, P=0.009) concentration. HOMA was not related to relative free fatty acid synthesis after higher fat intake, but was positively correlated with both 16:0 and 18:0 synthesis (both R=0.75, P=0.002). Insulin showed similar associations for lower fat intake, although the strongest relationship was with 14:0 concentration (R=0.73, P=0.003) and 18:0 synthesis (R=0.78, P=0.001). It was also correlated with the relative amount of total free fatty acids synthesized (R=0.67, P=0.009). Glucose was only significantly related to 14:0 concentration (R=0.66, P=0.009). The only other significant correlations for free fatty acid concentration were between BMI and 16:1 after both diets (lower fat R=0.84, P=0.0002, higher fat R=0.70, P=0.005) and 18:3 after higher fat intake (R=0.78 BMI, P=0.001). BMI was also positively correlated with the relative synthesis of several fatty acids in plasma, 16:0 (R=0.67, P=0.008), 18:0 (R=0.79, P=0.0007) and total relative free fatty acid synthesis (R=0.72, P=0.003), but only after lower fat intake.

Total Plasma Free Fatty Acids and Synthesized Free Fatty Acids

The relationships between VLDL-TG fatty acid concentrations (Chapter 4) and relative amounts of fatty acids synthesized in VLDL-TG (Chapter 5) were shown to be more numerous and stronger when subjects consumed the lower fat versus the higher fat diet. The opposite trend was observed for the relationship between free fatty acids synthesized and free fatty acid concentration, as outlined below.

Regarding free fatty acid synthesis, 18:0 was most commonly related to free fatty acid concentration. This relationship was significant for 16:1 (R=0.75, P=0.002) and total free fatty acid concentration (R=0.69, P=0.007) following lower fat intake. For higher fat intake, 18:0 synthesis was highly correlated with 16:0 (R=0.70, P=0.005), 18:0 (R=0.82, P=0.0004), 18:2 (R= 0.83, P=0.0003) and total free fatty acids (R=0.76, P=0.002).

Other correlations observed following higher fat intake but not lower fat included positive significant correlations between 16:0 synthesis and 18:0 (R=0.72, P=0.004), 18:2 (R=0.71, P=0.005) and total free fatty acid concentrations (R=0.67, P=0.009). Also, total relative free fatty acid synthesis was correlated with the plasma concentrations of 16:0 (R=0.67, P=0.009), 18:0 (R=0.69, P=0.006), 18:2 (R=0.77, P=0.001) and total free fatty acid (R=0.72, P=0.004).

When the relative amount of free fatty acid synthesized was compared for the fatty acids, 18:2 synthesis was not correlated with any fatty acid. 18:1 was less strongly correlated or not correlated at all with other fatty acids synthesized following lower fat intake but was highly positively correlated with relative synthesis of other fatty acids after higher fat intake, of which the strongest relationship was with total fatty acid synthesis (R=0.87, P=0.00004). Similarly strong positive relationships were observed between 16:0, 18:0 and total synthesis, the strongest of which was between 16:0 synthesis and total relative free fatty acid synthesis (R=0.93, P=0.000002). This confirms that the two are not only correlated, but that total synthesis must be highly dependent on 16:0 synthesis as net synthesis of 16:0 was much higher than that of any other fatty acid (Figures 6-2C). It would therefore contribute highly to total net synthesis of plasma free fatty acid.

Free Fatty Acid Concentration and VLDL-triglyceride Fatty Acid Concentration and Synthesis

Results of VLDL-triglyceride fatty acid concentration data from these subjects is shown in Chapter 4, and VLDL-TG fatty acid synthesis results are shown in Chapter 5.

Neither total nor any individual free fatty acid level in plasma was significantly correlated with total or individual VLDL-TG fatty acid concentration following lower fat intake. Following higher fat intake, strong correlations were observed between the concentrations of free 14:0 and VLDL-TG 14:0 (R= 0.67, P=0.008), free 16:1 and VLDL-TG 16:1 (R=0.80, P=0.0008), as well as both free 14:0 with VLDL-TG 16:1 (R=0.69, P=0.006) and free 16:1 with VLDL-TG 14:0 (R=0.70, P=0.006). No other significant relationships were found.

No relationships were evident between relative free fatty acid synthesis and VLDL-TG fatty acid concentration.

Free Fatty Acid Synthesis and VLDL-triglyceride Fatty Acid Synthesis

The relative synthesis of VLDL-TG 14:0 and 18:2 were not correlated with the relative synthesis of any free fatty acid. The relative amount of synthesized free 16:0 was the most highly correlated with the relative synthesis of all other fatty acids in VLDL-TG as well as total relative synthesis for both diets (R>71, P<0.01 for all), of which the strongest relationship was with VLDL-TG 16:0 (lower fat R=0.89, P=0.00002; higher fat R=0.86, P=0.00008). A similar pattern was observed for free 18:0 synthesis, of which the strongest relationship was with VLDL-TG 16:0 (lower fat R=0.80, P=0.0006; higher fat R= 0.82, P=0.0003).

The relative synthesis of individual fatty acids in VLDL-TG that were most closely related total relative free fatty acid synthesis were VLDL-TG 16:0 for both diets (lower fat R=0.70, P=0.005; higher fat R=0.79, P=0.0007), and 18:1 (R=0.69, P=0.007) and total (R=0.71, P=0.004) VLDL-TG fatty acid synthesis for the higher fat diet.

Free Fatty Acid Concentration and VLDL-triglyceride Fatty Acid Synthesis

Following lower fat intake, there were no significant correlations observed between the concentrations of total or individual plasma free fatty acids and the relative synthesis of total or individual fatty acids in VLDL-TG following lower fat intake.

After higher fat intake, the plasma concentrations of the following fatty acids were related to the relative synthesis of 16:0 in VLDL-TG: 16:0, 16:1, 18:0, 18:1, 18:3 and total free fatty acids. The weakest of these was 18:3 (R=0.70, P=0.005) and strongest was the correlation with total free fatty acids (R=0.82, P=0.0004). The relative synthesis of 16:1 in VLDL-TG was correlated with both 16:1 and total plasma free fatty acid concentration (R=0.68, P=0.008 and R=0.67, P=0.009, respectively). Total relative synthesis of fatty acid in VLDL-TG was correlated to the plasma concentrations of 16:0, 16:1, 18:0 and total free fatty acid (R>0.69, P<0.006 for all).

6.4 DISCUSSION

In Chapter 5, deuterium incorporation techniques were used to estimate hepatic fatty acid synthesis in non-diabetic control and diabetic subjects consuming short term lower and higher fat diets. Fatty acids synthesized in VLDL-TG were examined in relation to VLDL-TG fatty acid concentration and composition, and also to the synthesis of each fatty acid. Overall, diet had an effect on total VLDL-TG fatty acid synthesis and the synthesis of individual fatty acids, particularly myristic (14:0), palmitic (16:0) and palmitoleic (16:1) acids. Synthesis rates were highly variable, within subjects and particularly between subjects with some having propensity to overall higher synthesis rates. Both fasting and postprandial fatty acid synthesis seemed to be uniquely upregulated during lower fat intake in non-diabetic control subjects. Further, VLDL-TG fatty acid concentration but not total plasma triglyceride concentration was related to hepatic de novo fatty acid synthesis. The results from the current study show that the synthesis of plasma free fatty acids is also highly variable, and while meal intake suppresses fatty acid concentration in the blood, the proportion of fatty acids synthesized generally increased following a meal. Opposite to the effects observed in VLDL-TG, control subjects showed no change in fasting plasma fatty acid concentration. This is despite a significant effect of the higher fat diet in reducing relative synthesis compared to lower fat in both groups.

The contribution of the major fatty acids in plasma to total fatty acid composition remains remarkably close to equal for 16:0, 18:1 (oleic acid) and 18:2 (linoleic acid)(Figure 6-2A, Table 6-1). The levels for the entire group for both diets averaged 0.26±0.13, 0.23±0.06 and 0.23±0.08 mmol/L at 2h postmeal and 0.33±0.19, 0.39±0.15 and 0.29±0.12 mmol/L at 12h fasting, respectively. Scant research on plasma free fatty acid composition in humans (not of the plasma fatty acids but of the plasma non-esterified or "free" fatty acids) has reported similar levels but slightly different proportions (Aarsland & Wolfe, 1998). For instance 16:0, 18:1 and 18:2 were reported at 0.25±0.03, 0.43±0.04 and 0.12±0.03 mmol/L, which shows higher 18:1 and lower 18:2 proportions than in the current study. Interestingly, after 4 days of carbohydrate hyperalimentation, the total free fatty acid concentration did not change, however 18:1 increased and 18:2 decreased significantly from the basal state. This altered proportions of the major fatty acids, with concentrations reported at 0.26±0.00, 0.27±0.01 and 0.04±0.01 for 16:0, 18:1 and 18:2, respectively.

Another study, which reported 18:1 and 18:2 concentrations in plasma free fatty acids, indicated that 2h after a fat-rich meal, the levels of these two fatty acids were almost equal on average, at approximately 0.20 mmol/L. However changes over time were significant and depended on the fatty acid composition of the test meal as well as the previous meal (Fielding *et al*, 1996). In this study, chylomicron composition of 18:1 and 18:2 was very similar to the test meals composition, and plasma free fatty acid composition of 18:1 and 18:2 closely reflected that of the chylomicron fraction, leading to a the conclusion that lipoprotein lipolysis was contributing to the free fatty acid pool (Fielding *et al*, 1996). Indeed, other studies using isotope tracers have indicated that there is "spillover" of dietary fatty acids from chylomicrons into the plasma pool following a meal (Barrows & Parks, 2006). If there is a switching of substrate contributing to the free fatty acid pool during the postprandial period, then it should influence fatty acid profile, perhaps increasing the proportionate contribution of 18:1 after the higher fat/high oleate intake. However, this requires further exploration as this does not appear to be the case in the subjects from this study (Figure 6-4).

In contrast to the relatively equal levels of 16:0 and 18:1 in the non-esterified plasma pool, the fatty acid in this pool that incorporates deuterium label seems to be primarily 16:0 (Figure 6-2C). Relative synthesis, which is a fractional estimation of the amount of each de novo fatty acid as a % of the total pool of that fatty acid, seems to be more evenly distributed on average, with 14:0 and 16:0 at 7-20% de novo fatty acid, and 16:1 and 18:0 at around 2-5% de novo fatty acid (Figure 6-2B). Net synthesis is calculated when the relative synthesis is adjusted for the total amount of that individual fatty acid (de novo and non-de novo), and because the total 16:0 pool in plasma is much larger than that of 16:1 and 18:0, net synthesis in absolute contribution is much greater for this fatty acid.

It was difficult to examine overall trends in the synthesis data based on means, as the variation within and between subjects was appreciable. For instance, there were some subjects (n=4) that had no deuterium enrichment detectable above background levels in the plasma free fatty acids 24h after deuterium loading. This data was omitted from calculations of % contribution of individual fatty acids because there is no proportionate separation of individual fatty acids. One of the subjects showed no evidence of synthesis during fasting or postprandially for either diet, except 2 and 4h after the lower fat test meal, of which the net synthesis of total plasma fatty acid was 10 and 13mg, respectively. For those subjects that had some deuterium enrichment of plasma free fatty acids, the total amount of 24h net synthesis had a wide variation, ranging from 7 mg to 296 mg. Net synthesis within subjects also showed some variation, for instance, the smallest difference between lower to higher fat diet was 34 to 40 mg and the largest change was from 135mg to 0mg total de novo fatty acid. Relative synthesis of fatty acids did not vary as much, with a range over the entire group at 24h fasting of 0% to 9% of total fatty acids synthesized. Postprandial levels had a slightly higher maximum contribution reaching 14% relative synthesis in one subject. Comparatively, for relative synthesis within subjects the smallest range (with the exception of 0 to 0%) was no appreciable change at 9.2% to 8.8%, to a maximum change from lower to higher fat diet of 6.1% to 0% (same subject as maximum net change).

As previously outlined (6.2.3 Analytical Methods) the DNFAr or relative synthesis of total fatty acids is equal to the % contribution of synthesized or de novo fatty acid to the total pool. When considering the relative synthesis of individual fatty acids however, the %

synthesis is relative to the total pool of that individual fatty acid, not the entire plasma free fatty acid pool. In order to calculate the proportion (i.e. "proportional synthesis") of each de novo fatty acid in the total pool of de novo synthesized fatty acids, net synthesis was used to determine proportional synthesis. Overall, there was a high amount of variation, but on average each fatty acid contributed to the de novo pool as follows: 14:0 = 1±2%, 16:0 = 70±15%, 16:1 = 2±3%, 18:0 = 11±12%, 18:1 = 9±8% and 18:2 = 6±9%. As previously mentioned, the relative synthesis of 18:2 could be used as a gauge of "noise" in the method used (Figure 6-2B). Net synthesis of 18:2, however, does not represent noise as shown in Figure 6-2C, because the small relative percent of synthesis or noise will be be artificially inflated when net synthesis is determined as a result of the high concentration of this fatty acid in plasma (Equation 4 in 6.2.3 Anaytical Methods). Therefore, if one was to remove the "noise" from all synthesis data, the relative synthesis % of 18:2 would need to subtracted from the relative synthesis of all other fatty acids, before all other calculations are done. If it is true that 18:2 relative synthesis represents error of some sort, then it is conceivable that the actual synthesis of 18:1 and 18:0 is very minimal.

One of the objectives of this research was to determine if adipose tissue or intestinal de novo lipogenesis contributes to the plasma non-esterified pool of free fatty acids. As per the above discussion, it can be concluded that when deuterium is enriched in body water, deuterium label does appear in plasma free fatty acids. On a quantitative basis, most of the fatty acid labeled with deuterium in this pool is 16:0. However, despite this, de novo synthesized 16:0 only comprised from 5 to 14% of fatty acid in the total free plasma 16:0 pool. Since the plasma non-esterified pool of free fatty acids has only two primary sources, it can be surmised that one or both sources may be the origin of deuterium label. Specifically, fatty acids can be released into the bloodstream from adipose tissue through the action of hormone sensitive lipase and by lipolysis of triglyceride-rich lipoproteins by lipoprotein lipase. Since both of these sources can originate from exogenous and endogenous fatty acids through fatty acid recycling and de novo lipogenesis occurring in both intestinal and adipose tissue, some assumptions need to be made. The first assumption is that, due to the short duration of the deuterium loading, there will be minimal recycling of actual deuterium label over the study period. Plasma water enrichment levels show that deuterium was sufficiently washed out between diets (Figure 5-1). Also, in the formula for estimation of relative synthesis (DNFAr; 6.2.3 Analytical Methods Equation 2), both plasma water enrichment and background enrichment of the fatty acid are taken into consideration for each subject and adjusted. Further to this, previous research has indicated that during fasting, the primary source of plasma free fatty acids is adipose tissue. Following a meal lipoprotein lipolysis can comprise up to 44% of the fatty acid pool, the contribution of label in this 44% would be very minimal because it would be from intestinal de novo lipogenesis, which is thought to be negligible to begin with. Also, the possibility of recycling of label from lipolysed VLDL-TG is most likely also negligible. The assumption can therefore be confidently made that the source of the deuterium in total and individual free fatty acid in the plasma originated from adipose tissue lipolysis. It can also be assumed then, that if plasma non-esterified fatty acids have incorporated deuterium atoms from the body water, that the source of these labeled fatty acids is adipose tissue de novo fatty acid synthesis.

If these assumptions hold true, then published values using other methods of adipose tissue de novo lipogenesis should be comparable. Diraison et al. (2003) used a dual tracer method to separate labeling of hepatic and adipose tissue lipogenesis after acute and chronic changes in carbohydrate intake. Compared to liver, in which lipogenesis was both acutely and chronically stimulated by carbohydrate intake, adipose tissue responded poorly (chronic) or was not stimulated at all (glucose load). However, during unstimulated periods, adipose tissue and liver lipogenesis was estimated to be fairly comparable as estimated at 0.27±0.14g for adipose versus a net synthesis of liver fatty acid of 0.21±0.04g over the 12h testing period. With carbohydrate overfeeding, liver lipogenesis reached 0.58±0.22g, whereas adipose lipogenesis was as high as 0.41±0.19g on average (Diraison *et al*, 2003). Since it would appear that hepatic and adipose lipogenesis rates are similar, then it must also be assumed that perhaps plasma free fatty acid in this study can be used to determine the types of fatty acids primarily synthesized by adipose tissue, but not the quantitative contribution. It is likely that deuterium in adipose tissue fatty acid may take longer to incorporate into such a large pool and hence be released and measured as a reflection of adipose tissue fatty acid synthesis. It can still be assumed, however, that the source of the deuterium label in plasma free fatty acids in the current study is adipose tissue lipolysis.

An important question that needed to be answered was whether there was labeled fatty acids entering the liver fatty acid pool and therefore being incorporated into VLDL-TG, particularly in regards to the results from Chapter 5. If this was the case, then measuring deuterium incorporation in VLDL-TG could not be considered 100% "hepatic" fatty acid synthesis or there would need to be some sort of correction factor. According to the results from this study, there is potentially deuterium-labeled fatty acids entering the liver from the plasma free fatty acid pool, as these were highly correlated with the amounts of deuterium-labeled fatty acids in the VLDL-TG fatty acids, such as relative free fatty acid synthesis being highly correlated with VLDL-TG 16:0 for both diets. However, it can be concluded that this source is small for all fatty acids except 16:0. In comparing the net amount of de novo free fatty acid versus the net amount of de novo VLDL-TG fatty acid, the averages for the groups ranged from 21 to 76mg for free fatty acids and 204 to 526mg for VLDL-TG fatty acids. Clearly, if the labeled fatty acids reach the liver, the contribution can be considered quantiatively minor.

Another comparison that can be made and an objective of this research was regarding the contribution of total pool of plasma non-esterified fatty acids to VLDL-TG fatty acids. If plasma free fatty acids contribute to the synthesis of VLDL-TG, then amounts of total and individual fatty acids in the TG should correlate with amounts of total and individual fatty acids in the plasma pool. However, neither total nor any individual free fatty acid level in plasma was significantly correlated with total or individual VLDL-TG fatty acid concentration following lower fat intake. After higher fat intake, the only correlations observed were between the concentrations of the minor shorter chain fatty acids in plasma (14:0 and 16:1) and their concentration in VLDL-TG. Furthermore, the composition of free fatty acids seems to exhibit much less variation than VLDL-TG fatty acids. Despite that, the trend is towards a similar ratio of the major fatty acids in both fatty acid pools (Table 6-1). Since it can be concluded that the overall impact of lower fat intake on VLDL-TG fatty acid synthesis is upregulation, then it is possible that during increased hepatic lipogenesis and decreased supply of free fatty acid to the liver (i.e. 2h postmeal), the sources of fatty acid for VLDL-TG production may also change. If plasma non-esterified fatty acids are a major substrate for VLDL-TG synthesis (Lewis *et al*, 1995) and the availability of lipid is a major driver for VLDL production (Boren *et al*, 1991), then the lack of an obvious relationship between the plasma amount and composition of free fatty acids and the total amount and composition of the VLDL requires further investigation.

Table 6-1. Comparison of the ratios of palmitate : oleate : linoleate in the various fatty acid pools.

Source FA	**LF**	**HF**
VLDL-TGFA	1.6 : 2.3 : 1	1.8 : 3.3 : 1
FFA	1 : 1.2 : 1	1.1 : 1.4 : 1
DIET	0.4 : 1 : 1	0.6 : 3 : 1
H-DNL	40 : 8 : 1	28 : 6 : 1
A-DNL	9 : 1.3 : 1	15 : 1.6 : 1

Values are fasting ratios calculated from means of % composition data. LF: lower fat diet, HF: higher fat diet. TGFA: triglyceride fatty acid, FFA: plasma free fatty acid, H-: hepatic, A-: adipose, DNL: de novo lipogenesis.

Once fat reaches the liver it has many possible destinations, such as oxidation, incorporation into phospholipids (i.e. lipoproteins), conversion to other fatty acids and metabolites, transport out of the liver as VLDL-TG, or storage in the cytosolic pool. If there is any variation in the proportion of each fatty acid in VLDL-TGFAs from the proportion in free fatty acids, then two situations are possible. That is, the partitioning of individual fatty acids towards one of these pathways, or the dilution of liver triglycerides with fatty acids from other sources. Hepatic lipogenesis and dietary input from chylomicron remnant uptake or lipolysis has the potential to influence fatty acid composition. The higher oleate proportion in the lower fat VLDL-TG fatty acid would indicate that dietary input had an influence. Alternatively, the high palmitic acid composition of de novo fatty acids could potentially influence the amount of palmitic acid in VLDL-TG fatty acid composition.

Alternatively, the relative synthesis of free fatty acids can be tested for correlation with the synthesis of other free fatty acids. This may aid in determining if label from one fatty acid is associated with label in other free fatty acids. In other words, a high synthesis rate of 14:0

may be correlated with a high amount of label in 16:0 because it is used as a primer for 16:0 elongation. Indeed, correlation data showed a strong positive relationship of de novo fatty acids with other de novo fatty acids, possibly indicating that if one fatty acid is highly synthesized, then the elongation product of that fatty acid is also likely to be highly synthesized from the shorter chain fatty acid as a primer. Alternatively, it may simply be an indication that synthesis of all fatty acids is upregulated to an equal extent. The scope of this research is not able to answer these questions definitively, and further research is required to explore this more specifically.

Although there were no significant effects of diabetes on plasma free fatty acid concentration or synthesis, there seems to be a relationship with insulin levels and insulin resistance. Previous research has shown a complicated relationship of of insulin resistance with free fatty acids concentration, but a causal relationship has never been proven in vivo.

6.5 CONCLUSION

For the first time in human subjects, it was found that using deuterium incorporation techniques to estimate hepatic de novo lipogenesis may result in some incorporation of deuterium from the free fatty acid pool. The exact contribution to deuterium in hepatic de novo fatty acid could not be determined, but can be considered minor compared to the total amount of fatty acid synthesized in the liver. A comparison of the fatty acid composition of the various pools that could influence the fatty acid profile of VLDL-triglyceride was also determined, which has previously never been reported. The results from this research indicate that the synthesis of plasma free fatty acids is highly variable, and while meal intake suppressed fatty acid concentration in the blood, the proportion of free fatty acids synthesized generally increased following a meal. Diet affected the synthesis and concentration of the non-esterified fatty acid pool in these subjects more so than the presence of diabetes, with a large variation in total synthesis and response to diet. In examining the fatty acid composition of plasma free fatty acids in comparison to VLDL-TG fatty acids, it is conceivable that this fatty acid pool is a major contributor, with diet and lipogenesis also affecting composition.

6.6 REFERENCES CITED IN CHAPTER 6

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CHAPTER 7

THESIS CONCLUSIONS AND FUTURE DIRECTIONS

7. 1 CONCLUSIONS

7.1.1 EXPERIMENT 1

Hypothesis 1 (Chapter 3): ***Lowering carbohydrate intake by replacement of calories from carbohydrate with monounsaturated fat will reduce plasma TG levels and hepatic de novo lipogenesis. This will have a greater effect in subjects with higher baseline TG and diabetic subjects, and should not adversely affect weight or cholesterol levels.***

This study was designed to mimic the free-living situation where small changes in dietary intake occur within the scope of an individual's habitual diet. Subjects were counseled to increase fat intake by incorporating visible olive oil into their usual diet, while isocalorically reducing carbohydrate-rich foods. Seven-day food records were used to detect dietary fat intake change of >5% of energy. This exchange of carbohydrate and fat calories resulted in a consistent TG-lowering effect which was similar for both diabetic and non-diabetic subjects. The trend towards a decrease in plasma TG concentrations was significant in a subgroup of subjects with higher TG when consuming lower fat. Contrary to the hypothesis, the changes measured in hepatic fatty acid fractional synthetic rate did not correspond to changes in plasma triglyceride concentration in this group of subjects. However there was a significant positive relationship between hepatic fatty acid synthesis and plasma triglyceride concentration in control subjects that was not evident in type 2 diabetes subjects.

The results of this study indicate that higher fat intake achieved by increasing olive oil consumption could have beneficial effects on fasting plasma TG concentrations or borderline hypertriglyceridemia in type 2 diabetes subjects. The single fasting measure may not have fully captured the contribution of de novo lipogenesis to postprandial lipemia. Further research is required to delineate the role of dietary fat and carbohydrate to postprandial TG and the contribution of hepatic fatty acid synthesis in subjects with type 2 diabetes.

7.1.2 EXPERIMENT 2

Hypothesis 2 (Chapter 4): ***Diabetes and/or lower fat intake will result in higher fasting and postprandial plasma triglycerides and these differences will be reflected in VLDL-triglyceride levels. It is specifically hypothesized that a higher composition of palmitate and stearate and lower composition of oleate will characterize the fatty acid composition differences between diabetes/lower fat intake and non-diabetes/high oleate intake, perhaps due to effects of fatty acid composition on production or clearance of VLDL-triglyceride.***

This study compared the effect of higher versus lower fat intake on postprandial triglyceride levels and VLDL-triglyceride levels in diabetic and non-diabetic subjects. VLDL-triglyceride fatty acid composition was also examined in response to diet changes. Type 2 diabetes subjects were matched with non-diabetic respondents and fed two diets for 3 days in a blinded randomized crossover design and one-month washout period. Diets consisted of identical items differing in energy from fat (lower fat <25%; higher fat >35% achieved by addition of canola oil). It was found that the small reduction in TG concentration after higher fat intake was consistent with a significant reduction in VLDL-triglyceride. In non-diabetic control subjects, all of the fatty acids that were measured during fasting decreased on average. Myristic, palmitic and palmitoleic acids showed significant reductions, even though levels of these fatty acids were held constant between diets. There was a significant effect of diabetes on total postprandial triglycerides indicating that postprandial lipemia seems [illegible] be of a greater magnitude in diabetic subjects. Postprandial levels of individual fatty acids were highly variable, however the diabetes group generally seemed to have higher myristic acid in VLDL-triglyceride, related to insulin resistance. The control group appeared to consistently increase 16 carbon fatty acids when consuming the lower fat diet. It is possible that de novo lipogenesis may have been contributing to these pools and that insulin resistance specifically affected elongation of myristate to the 16 carbon fatty acids. Further investigation is required to identify the role of de novo lipogenesis in VLDL-triglyceride fatty acid composition. From this research, it appears that differences in VLDL-TG fatty acid composition may depend on both dietary fat level and the presence of insulin resistance.

Hypothesis 3 (Chapter 5): ***Hepatic de novo synthesis of total and saturated fatty acids is higher in diabetic subjects and also after lower fat/higher carbohydrate intake, resulting in differences in VLDL-triglyceride fatty acid composition.***

Deuterium incorporation techniques were used in the same subjects as outlined above and hepatic de novo synthesis of total and individual fatty acids was estimated. This technique has never been used in the estimation of de novo synthesis of individual fatty acids.

There was an effect of diet on 24h fasting fractional synthetic rate of fatty acids in VLDL-triglyceride. Relative de novo fatty acid synthesis and net synthesis of total and individual fatty acids showed a wide range of variation, between diets and between subjects. Fatty acid synthesis was upregulated in non-diabetic control subjects following short term lower fat intake, despite the low glycemic index/high fibre content of this diet. This overall increase in hepatic fatty acid synthesis was not observed in diabetes subjects, contrary to the original hypothesis. Estimates of fasting and postprandial lipogenesis showed that although the average relative contribution of de novo fatty acid may only range from 4 to 19% of total VLDL-triglyceride, in individual subjects this amount can range from 0 to 48%. This could potentially produce up to 18g of de novo fatty acid per day, 16 of which would be saturated fatty acids. Palmitate was the major product of de novo lipogenesis, comprising an average of 68% of total de novo fatty acids. Furthermore, regardless of diet, palmitate synthesis was highly correlated with total hepatic fatty acid synthesis and thus measuring palmitate synthesis may reflect total synthesis. However an underestimation may occur in some cases as the proportion of total de novo fatty acid from palmitate ranged from 44 to 84% in these subjects. Lipogenesis, which is greatly upregulated in some individuals, produces fatty acids that are more saturated, potentially affecting the overall composition of triglyceride in the VLDL. It appears that dietary fat amount is an important determinant of changes in de novo lipogenesis, however in diabetes subjects this effect may be blunted. The effects of variations in synthesis of total and saturated fatty acids in individual subjects may be of particular importance to risk of metabolic disease and this area of research requires further examination.

Hypothesis 4 (Chapter 6): ***Plasma free fatty acid composition is similar to VLDL-triglyceride fatty acid composition as these are the main source of fatty acids for VLDL-triglyceride synthesis. There may be a small contribution of intestinal or adipose tissue de novo fatty acid to VLDL-TG synthesis, and this will be detected in plasma free fatty acids through the release of labeled fatty acids from adipose tissue triglyceride lipolysis or spillover from chylomicron triglyceride lipolysis.***

Deuterium incorporation techniques were used in the same subjects as outlined above and total and individual de novo free fatty acid in plasma was estimated. Deuterium enrichment of plasma free fatty acids after a loading dose of deuterium oxide has never been used to estimate de novo synthesis of total or individual fatty acids in the plasma non-esterified fatty acid pool.

The results from this research indicate that synthesis of plasma free fatty acids is highly variable, and while meal intake suppressed fatty acid concentration in the blood, the proportion of free fatty acids synthesized generally increased following a meal. No differences were detected between diabetes and control subjects, with diet having a similar effect on synthesis of free fatty acid overall. The contribution of major fatty acids in plasma to fatty acid composition was consistently close to equal for palmitate, oleate and linoleate, however de novo synthesis of fatty acid was comprised almost solely of palmitate. Synthesis of other fatty acids occurred in most subjects, but the total pool was small and did not contribute substantially to net total synthesis. There was a large variation in total synthesis and response to diet. Some subjects showed no detectable synthesis, while others exhibited a net synthesis of up to 296mg at a relative synthesis of 9% of the total free fatty acid pool. In response to diet change, some subjects exhibited no change in synthesis and others experienced a 300% decrease or complete suppression of synthesis after higher fat intake. In examining the fatty acid composition of plasma free fatty acids in comparison to VLDL-TG fatty acids, it is conceivable that this fatty acid pool is a major contributor, with diet and lipogenesis also affecting composition. It was also determined that deuterium labeled fatty acids from the plasma pool (adipose tissue de novo lipogenesis) may contribute to deuterium labeled fatty acids in VLDL-triglyceride (hepatic de novo lipogenesis) in some subjects, however it is likely a quantitatively insignificant amount.

7.2 SUMMARY OF FINDINGS

Overall, it was expected that alterations in triglyceride metabolism during high carbohydrate intake or in insulin resistance may differ from that resulting from higher fat diets. Interestingly, diabetes and diet have unique effects on plasma triglyceride concentration and fatty acid composition. Diabetes and insulin resistance tended to affect aspects of fasting and postprandial concentration, whereas diet influenced fatty acid composition more profoundly. Higher monounsaturated fat/lower carbohydrate intake did not increase postprandial triglycerides and generally decreased VLDL-triglyceride and de novo lipogenesis. Higher glucose/insulin levels related to many aspects of fat metabolism, particularly the concentration and synthesis of shorter chain fatty acids. Palmitate was the major product of de novo lipogenesis, although contribution to both total synthesis and the total fatty acid pool varied considerably. In fact, variability of lipogenesis and differences between subjects in response to dietary change may be one of the most important findings of this research. At the rate of lipogenesis observed in some subjects, this source of fatty acids in VLDL-triglyceride has the potential to affect both the quantity of triglyceride and fatty acid composition, potentially increasing the amount of circulating saturated fatty acids. Whether these differences in lipogenic capacity contribute to hypertriglyceridemia and other metabolic alterations that occur in diabetes is still unknown.

7.3 SIGNIFICANCE OF RESEARCH

Findings from this research will contribute to understanding the differences in fatty acid composition between individuals and in response to dietary composition. Previous research has been highly focused on fatty acid composition of diet and disease risk. This is obviously important; however it is now becoming clear that dietary composition of carbohydrate is also important, particularly in some individuals. While dietary fat composition has been shown to affect disease risk, it is more likely that how nutrients are processed in the body may be even more detrimental. Many genes influence lipid transport, synthesis, oxidation, conversion to other fatty acids and metabolites, and the research surrounding the unique combination of these genes in individuals and modulation of the expression of these genes through diet is still in its infancy.

Diabetes has reached epidemic proportions around the world and is expected to increase as the population ages and becomes more obese. Nutrition may have an important role to play in influencing the regulation of hepatic lipid processing, and therefore the potential to affect the prevalence and health consequences of dyslipidemia, insulin resistance, diabetes and cardiovascular disease. Current technology for following human lipid metabolism using stable isotopes is becoming more advanced, as shown by the results of this thesis research. The importance of in vivo study of synthesis, assembly, and secretion of triglycerides in humans is principal to fully understanding the pathology of altered lipid metabolism in diabetes and the role of diet, as well as in determining preventative strategies.

7.4 FUTURE DIRECTIONS

It has now been established that fatty acid synthesis occurs to a significant extent in some individuals, in amounts that could potentially affect fatty acid composition and result in the accumulation of fat and particularly saturated fatty acids in the body. Although fatty acids synthesized follow certain trends, these trends can vary substantially between individuals and dietary intake. Identifying specific changes that occur in individuals and the trends that occur as a result of diet and insulin resistance are needed. Methods surrounding deuterium incorporation techniques had not yet been utilized in combination with the capabilities of the gas chromatograph pyrolysis mass spectrometer. Therefore a re-evaluation of the raw data, how it can be processed and calculated and the re-examination of the results will be a worthy future effort.

Future research will have a major role in delineating whether differences in fatty acid composition and lipogenic capacity increase the risk of metabolic disease such as metabolic syndrome and diabetes. It will be important to determine what factors are associated, both genotypic and phenotypic, and if modulation of these factors can mitigate disease risk. The effects of lower fat/higher carbohydrate diets on all aspects of lipid and lipoprotein metabolism need to be established in order to determine whether these diets actually reduce cardiovascular disease risk. Carbohydrate-induced hypertriglyceridemia remains a complicated process that research is just beginning to unravel.